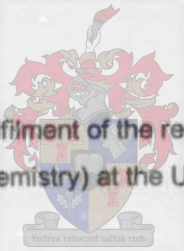
INVESTIGATIONS INTO THE PRODUCTION OF A HARPIN ELICITOR BY *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE* ISOLATED FROM A NECTARINE TREE

Maryke Appel



Thesis presented in partial fulfilment of the requirements for the degree of Master of Science (Biochemistry) at the University of Stellenbosch

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March 1996

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

1996-02-19
Date

For my parents

SUMMARY

Bacterial canker of stone fruit trees, caused by *Pseudomonas syringae* pv. *syringae*, has become one of the most destructive crop diseases in South Africa. Failure of chemical control of the disease has rendered the selection and breeding of resistant host trees an important aspect of future control strategies. To assist in such breeding programmes, investigations into the molecular basis of the host-pathogen interaction were initiated.

The fundamental ability of phytopathogenic pseudomonads, xanthomonads and non-soft rot erwinias to cause necrotic diseases in their hosts and hypersensitivity in non-host plants is controlled by their widely conserved *hrp* gene clusters. The only known secreted *hrp* gene product, dubbed "harpin", has been identified as the molecule ("elicitor") both responsible and required for eliciting hypersensitivity or disease symptoms.

In this study, the production of a harpin elicitor by a strain of *Pseudomonas syringae* pv. *syringae*, isolated locally from a nectarine tree (*P. s.* pv. *syringae* NV) was investigated. The HR test in tobacco was used to assess the elicitor activity of bacterial fractions. It was established that the bacterium produces an extracellular protein elicitor similar to harpin$_{Pss}$, the harpin elicitor of the wheat and bean pathogen, *Pseudomonas syringae* pv. *syringae* 61. Antibodies were raised against harpin$_{Pss}$ and used to confirm homology between the elicitors of the two strains, using Western blot analysis.

Homology between the two proteins was exploited on the gene level in the design of a polymerase chain reaction strategy for the amplification of the harpin encoding gene of *P. s.* pv. *syringae* NV from its genomic DNA. Partial sequencing of the single PCR product and Southern blot hybridization with a probe based on the *P. s.* pv. *syringae* 61 harpin encoding gene, confirmed its identity as the harpin encoding gene of *P. s.* pv. *syringae* NV.

OPSOMMING

Bakteriese kanker van steenvrugte, wat deur *Pseudomonas syringae* pv. *syringae* veroorsaak word, is tans een van die mees verwoestende siektes van landbougewasse in Suid-Afrika. Die mislukking van chemiese beheermaatreëls het die seleksie en teling van weerstandbiedende gasheerbome 'n belangrike aspek in toekomstige beheerstrategieë gemaak. Studies wat die molekulêre basis van die gasheer-pathogeen interaksie ondersoek, is van stapel gestuur om tot sulke teelprogramme by te dra.

Die fundametele vermoë van sekere bakterieë om nekrotiese siektes in gasheerplante en hipersensitiwiteit in nie-gasheerplante te veroorsaak, word deur hul wyd-gekonserveerde *hrp* gene beheer. Die enigste bekende uitgeskeide *hrp* geenproduk, wat "harpin" genoem word, is geïdentifiseer as die molekuul ("elisitor") wat verantwoordelik is en vereis word vir die ontlokking van hipersensitiwiteit of siektesimptome.

In hierdie studie is die produksie van 'n "harpin" elisitor deur 'n ras van *Pseudomonas syringae* pv. *syringae*, wat plaaslik vanaf 'n nektarienboom geïsoleer is (*P. s.* pv. *syringae* NV), ondersoek. Die HR-toets in tabak is gebruik om die elisitor aktiwiteit van bakteriële fraksies te bepaal. Daar is vasgestel dat die bakterium 'n ekstrasellulêre proteïen elisitor produseer wat soortgelyk is aan harpin$_{Pss}$, die "harpin" elisitor van die storing- en boontjiepatogeen, *Pseudomonas syringae* pv. *syringae* 61. Antiliggame is teen harpin$_{Pss}$ opgewek om homologie tussen die elisitors van die twee rasse te bevestig, deur van die Western-klad tegniek gebruik te maak.

Homologie tussen die twee proteïene is op geen-vlak uitgebuit om 'n polimerase kettingreaksie-strategie te ontwerp waardeur die "harpin" koderende geen van *P. s.* pv. *syringae* NV vanuit sy genomiese DNA geamplifiseer kon word. Gedeeltelike volgordebepaling van die enkele PKR-produk en Southern-klad hibridisasie met 'n gedeelte van die *P. s.* pv. *syringae* 61 "harpin" koderende geen, het bevestig dat die PKR-produk die "harpin"-koderende geen van *P. s.* pv. *syringae* NV is.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to:

My supervisor, **Dr Dirk Bellstedt**, for his interest, patience, advice and encouragement throughout the study and in the preparation of this thesis, and for the opportunity to present my work at the Fifth International Conference on *Pseudomonas syringae* pathovars and related pathogens in Berlin, Germany.

Dr Lucienne Marsvelt, for her help, enthusiasm and encouragement throughout the study.

My Biochemistry lecturers, **Prof K J van der Merwe**, **Prof J-H S Hofmeyr**, **Prof P Swart**, **Dr D U Bellstedt**, **Mr E J F Foster**, **Ms A Louw** and **Ms M Rautenbach** for training me to discover and appreciate life on a molecular level.

Ms Amanda Swart, **Ms Coral de Villiers** and the late **Mr De Wet Ries** for their willingness to share their expert practical experience.

Mr Benno Rehder for always being prepared to get hold of the impossible ... yesterday.

The technical staff at the Department of Biochemistry, **George**, **Kerneels**, **Welma** and **Anita** and the technical staff at Infruitec, **Lily** and **Veronica**, for their daily contribution towards smoothing laboratory logistics.

Fellow postgraduate Biochemistry students, **Erika**, **Jolanda**, **Craig**, **Arrie**, **Wynand**, **Caz**, **Yolanda**, **Stephan** and **André** their encouragement, discussions, debates and social support.

Mr Mathias Hampf for his help with sequencing experiments.

The **Division of Plant Biotechnology and Pathology, Infruitec**, for the opportunity to undertake and continue with this research.

The **FRD**, **University of Stellenbosch** and **Harry Crossley Foundation** for financial support in the form of bursaries for this study.

Gencor, for financing my undergraduate studies and for their interest in and support of my postgraduate studies.

My parents for having me, raising me to question everything and loving and supporting me regardless.

***Pseudomonas syringae* pv. *syringae* NV** for fascinating me, frustrating me and keeping me humble.

ABBREVIATIONS

A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
A_{620}	Absorbance at 620 nm
avr	Avirulence
bcCSN	Boiled concentrated culture supernatant
bp	Base pairs
bP	Boiled pellet
bSSN	Boiled sonication supernatant
cCSN	Concentrated culture supernatant
cfu	Colony forming units
CSN	Culture supernatant
DIG	Digoxigenin
dNTP	Deoxynucleotide triphosphate
ddNTP	Dideoxynucleotide triphosphate
EPS	Extracellular polysaccharides
HR	Hypersensitive response
hrm	Hypersensitive reaction modulation
hrp	Hypersensitive reaction/pathogenicity
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	kilobase pairs
kDa	Kilodalton
LB	Luria-Bertani
MES	2-N-morpholino-ethanesulfonic acid
M_r	Molecular mass
NB	Naked bacteria
ND-PAGE	Non-denaturing polyacrylamide gel electrophoresis
NYGB	Nutrient-yeast extract-glycerol broth
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonylfluoride
pv.	Pathovar
R_f	Relative mobility
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SP	Sonication pellet
SSN	Sonication supernatant
TCA	Trichloroacetic acid
T_m	Melting temperature
Tris	Tris(hydroxymethyl)-aminomethane
UV	Ultra-violet
v/v	Volume per volume
w/v	Weight per volume
XR	Ionic exchange response

CONTENTS

CHAPTER 1

INTRODUCTION

Bacterial canker of stone fruit (peaches, nectarines, apricots, plums, almonds and cherries) has become one of the most destructive crop diseases in South Africa. Stone fruit growers in the Western Cape and cherry-producing region of the eastern Orange Free State are particularly affected and their losses in terms of diseased trees and reduced production are estimated to exceed R 25 million annually. *Pseudomonas syringae* pathovar *syringae* and *P. s.* pv. *morspronorum* have been identified as the causative agents of the disease, the former being the most general and widespread. Although the disease is not unique to South Africa, it is more severe in this country than elsewhere in the world. Environmental and horticultural practices have been implicated in this phenomenon, as well as in the failure of conventional control strategies. Some of these predisposing factors can, however, not be remedied and future control strategies will have to rely strongly on the breeding of resistant host trees. To assist in such programmes, investigations into the molecular basis of the interaction between *P. syringae* and stone fruit trees have been initiated (Roos and Hattingh 1983a, 1986a, 1987a, Hattingh *et al.* 1989).

The work of Klement and co-workers during the early sixties marked the beginning of investigations into the molecular basis of interactions between phytopathogenic bacteria and plants. Their widely quoted experiments, involving the artificial infiltration of tobacco leaves with a variety of phytopathogenic and saprophytic *Pseudomonas* species (Klement 1963, Klement *et al.* 1964), distinguished three types of plant-bacterium interactions:

1. A compatible interaction, which occurs specifically between a plant pathogen and a susceptible host plant (e.g. *P. s.* pv. *tabaci* and tobacco) and leads to the expression and spread of disease symptoms over a period of 2-5 days.

2. An incompatible interaction, which develops non-specifically between phytopathogens and non-host plants or resistant host plants and leads to the expression of a so-called "hypersensitive response" (HR) within 12-48 hours. These reactions, typically characterized by localized necrotic lesions, were

observed when a variety of *P. syringae* pathovars (other than *P. s.* pv. *tabaci*) were infiltrated into tobacco.

3. No observable interaction, which occurred when the saprophytic *P. fluorescens* was infiltrated into tobacco.

Subsequent studies confirmed the ability to elicit the HR to be a unique characteristic of a large group of plant pathogenic bacteria (the phytopathogenic pseudomonads, xanthomonads and non soft-rot erwinias) (Sigee 1993). Numerous studies have been dedicated to the elucidation of the nature and sequence of HR-associated events in both the pathogen and plant, in an attempt to define the relationship between pathogenicity and hypersensitivity (reviewed in Klement 1982, Sigee 1993, Goodman and Novacky 1994). Nevertheless, many aspects of this relationship remained vague until the mid-eighties, when it was discovered that a common gene system, designated the *hrp* genes, controls the development of both phenomena (Lindgren *et al.* 1986).

Even before this discovery, the actual HR eliciting bacterial factor was predicted to be an extracellular protein (Sasser 1980). This was confirmed by studies on the composition and regulation of the *hrp* gene cluster (reviewed in Hutcheson *et al.* 1994). However, a secreted bacterial protein elicitor (harpin$_{Ea}$, that of the apple and pear fire blight pathogen, *Erwinia amylovora* strain Ea321), was only isolated successfully after over-expression of its corresponding gene (*hrpN*) in a recombinant system (Wei *et al.* 1992a). This achievement was repeated shortly thereafter with the isolation and characterization of harpin$_{Pss}$, product of the *hrpZ*[1] gene of *P. s.* pv. *syringae* strain 61 (isolated from wheat, but also weakly pathogenic on bean; Atkinson and Baker 1987) by He *et al.* (1993). Functional expression of these proteins were shown to be essential for the development of both pathogenicity and hypersensitivity. Although purified harpin preparations are not sufficient for the development of disease symptoms in host plants, they elicit a hypersensitive response in non-host plants. For this reason they have become a valuable tool in the

[1]Further characterization of the *P. s.* pv. *syringae* 61 *hrp* gene cluster has led to the renaming of some of the loci. The harpin$_{Pss}$ encoding gene (referred to as *hrpZ* in the pre-1994 literature) has been identified as the second open reading frame (ORF) of a polycistronic operon. The operon was subsequently named *hrpZ* and the harpin$_{Pss}$ ORF *hrpZ2* (Xiao and Hutcheson 1994). This new designation will be used throughout the thesis.

study of plant host-pathogen interactions on the molecular level (Wei *et al.* 1992a, He *et al.* 1993).

This study was undertaken upon request of and in collaboration with the Agricultural Research Council's Institute for Fruit Technology (Infruitec) in Stellenbosch. It represents one of the first molecular investigations into the causative agent of bacterial canker in South Africa and comprises an investigation into the production of a harpin elicitor by *Pseudomonas syringae* pv. *syringae* isolated from a nectarine tree. This strain, designated *P. s.* pv. *syringae* NV, was isolated by Dr. E.L. Mansvelt of Infruitec in 1983 and was the first strain of the pathogen to be isolated locally from the *fruit* of an affected stone fruit tree (E.L. Mansvelt, personal communication). The objectives of the study were (a) to isolate and purify the nectarine pathogen elicitor and (b) to try and locate the gene encoding the nectarine pathogen harpin for cloning purposes. It was envisaged that this would form the basis of a comprehensive project investigating the application of a purified harpin in the design of a system to screen commercially important stone fruit cultivars for resistance against bacterial canker on a biochemical basis.

In this thesis, perspectives on the study field and motivations for research approaches are presented in the following two chapters. An overview of the interaction between *P. s.* pv. *syringae* and stone fruit trees in given in chapter 2. The chapter is introduced with general aspects of the phytopathogenicity of *P. syringae*, and provides the necessary framework in which to address the South African bacterial canker scenario and local research priorities. The *hrp* gene cluster and harpin$_{Pss}$ of *P. s.* pv. *syringae* 61 are discussed in chapter 3, as models upon which investigations in this study were based. The use of the HR test as a bioassay for pathogenicity/elicitor activity is also discussed briefly.

Experimental work is presented in chapters 4 and 5. Each of these chapters is written in the form of an independent publication. The identification of a harpin elicitor produced by *P. s.* pv. *syringae* isolated from a nectarine tree, using antibodies against harpin$_{Pss}$ of *P. s.* pv. *syringae* strain 61, is described in chapter 4. This work was presented as a poster at the Thirty-third Annual Congress of the Southern African Society for Plant Pathology (15-18 January 1995, Thaba 'Nchu)

and at the Thirteenth Congress of the South African Biochemical Society (2-5 April 1995, Bloemfontein). The amplification and characterization of a *hrpZ*2-like DNA fragment from the genome of *P. s.* pv. *syringae* NV is presented in chapter 5. This part of the study was presented as a poster at the Fifth International Conference on *Pseudomonas syringae* pathovars and related pathogens (3-8 September 1995, Berlin, Germany). Consequently, a shortened version of chapter 5 will appear in the proceedings of this conference, to be published in 1996.

Although discussions of experimental aspects are included in each of chapters 4 and 5, the thesis is concluded with a general discussion (chapter 6) of the approaches followed in this study, the fulfilment of objectives and future prospects in this research field in South Africa. To eliminate duplication, a list of cited references is not included in each of the experimental chapters, but a comprehensive reference list is given at the end of the thesis.

CHAPTER 2

THE INTERACTION BETWEEN *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE* AND STONE FRUIT TREES: AN OVERVIEW

1. The phytopathogenicity of *Pseudomonas syringae*

Very few bacterial species have evolved the ability to cause disease in plants (Chatterjee and Vidaver 1986). Lists published by the American Phytopathological Society (Hansen 1985, Smiley 1988) indicated that only 5-10 % of named diseases of agricultural and ornamental crops are attributed to phytopathogenic *bactena* (compared to 50-65 % attributed to fungi and 10-20 % to viruses). Nevertheless, bacterial diseases of crop plants are the major cause of financial losses to farmers in many parts of the world (reviewed in Sigee 1993).

The genus *Pseudomonas* comprises one of the five major taxonomic groups containing all phytopathogenic bacteria (the others being the genera *Agrobacterium*, *Erwinia*, and *Xanthomonas* and the coryneform bacteria (*Arthrobacter*, *Clavibacter*, *Curtobacterium* and *Nocardia* and *Rhodococcus*); Young *et al.* 1992, Sigee 1993). A detailed account of the classification of the genus *Pseudomonas* falls beyond the scope of this study. The reader is referred to Palleroni (1984) for this purpose. Apart from plant pathogens, the genus also contains a number of animal and human pathogens, as well as non-pathogens. The last three groups are omitted from further discussions.

1.1. Classification of phytopathogenic pseudomonads

The phytopathogenic pseudomonads are a very heterogeneous group of bacteria with regard to their genetics, ecology and the nature of the diseases caused by them. They are currently grouped in some twenty-five species, many of which are (still) inadequately characterized (Schroth *et al.* 1992). As a result, only thirteen phytopathogenic species of *Pseudomonas* were included in the *Approved Lists of Bacterial Names* (Skerman *et al.* 1980, update: Moore *et al.* 1985). To circumvent this problem, an interim scheme was developed, by which many distinct pathogens

were designated as "pathovars" of recognized species. The term *pathovar* was to be "used to refer to a strain or set of strains with the same or similar characteristics differentiated at infrasubspecific level from other strains of the same species or subspecies on the basis of distinctive pathogenicity to one or more host plants" (Dye *et al.* 1980). According to this criterium, three pathovars of *P. marginalis*, two of *P. gladioli* and forty-one of *P. syringae* were cited in the 1980 pathovar list. However, more recent publications (e.g. Guillorit and Samson 1993a) state the number of pathovars of *P. syringae* to be "about fifty".

Despite the interim arrangement, diversity within the *P. syringae* group has sustained an ongoing debate over the taxonomy of this species. Distinction based primarily on host range proved to be problematic, as (a) overlapping host ranges occur, and (b) it provided no evidence on the phenotypic and genotypic relationship between pathovars (Schroth *et al.* 1992). Diversity among pathovars in the species is not limited to host range, but has also been demonstrated on the genotypic level; studies dating back as early as 1972 indicated DNA-DNA homologies within the *P. syringae* group to range from 37 to 100 % (Palleroni *et al.* 1972, Pecknold and Grogan 1973, Denny 1988). This predicted the phenotypic heterogeneity noted for this species in *Bergey's Manual of Systematic Bacteriology* (Palleroni 1984), where many of the presented tests are listed as being "variable" for the various taxa.

Over and above the classic morphological, physiological and biochemical criteria employed in bacterial taxonomy, many techniques (reviewed extensively in Austin and Priest 1986, and Sigee 1993) have been devised and employed to elucidate the relationships between pathovars in the *P. syringae* group. To this end, a system of three-dimensional modelling of various bacterial characteristics, including genotypic, phenotypic and pathogenicity data, was developed by Hildebrand *et al.* (1982, 1984, 1987, 1988). Nevertheless, efforts in this regard continue and papers suggesting relationships and/or the re-classification (or renaming) of pathovars are published every year (see Gardan *et al.* 1991, 1992a, Hendson *et al.* 1992, Guillorit and Samson 1993b, Legard *et al.* 1993, Wiebe and Campbell 1993, Bereswill *et al.* 1994, Scholz *et al.* 1994, Mugnai *et al.* 1994, Ovod *et al.* 1995 for the most recent examples). At the Fifth International Conference on *Pseudomonas syringae* pathovars and related pathogens, held recently in Berlin, Germany, a re-evaluation

of the classification of the species and its pathovars was identified as one of the priorities in this research field.

1.2. Host range of Pseudomonas syringae

The recognition of more than forty *P. syringae* pathovars implies (by definition) that the species has an extensive host range. The pathovar list of Dye *et al.* (1980) included references to the reports in which each of the pathovars was first described (dating from 1893, for *P. s.* pv. *mori*, to 1972 for *P. s.* pv. *ciccaronei*). Upon comparison with the literature it is evident that:

(a) host plants include a wide variety of agricultural and ornamental crop plants (of which many are of great economic importance in different regions of the world), and

(b) according to their definition, the majority of individual pathovars have a specific and narrow host range (i.e. are only known to attack the "original" host and closely related plants). Some exceptions are: (i) both pathovars *tabaci* and *mellea* were originally isolated from tobacco (Wolf and Foster 1917, Johnson 1923), (ii) pv. *tabaci* has been reported to also cause disease of soybean (Kennedy and Tachibana 1973), (iii) some strains of pathovar *phaseolicola* (originally isolated from bean) are known to also infect mulberry and soybean (Schroth *et al.* 1971) and (iv) pv. *tomato* has been isolated from diseased stone fruit trees (Schroth *et al.* 1992).

The one pathovar that defies the pathovar definition (Dye *et al.* 1980) completely, is *P. s.* pv. *syringae* (originally isolated from lilac (*Syringa vulgaris* L.) by Van Hall, 1902). This bacterium has been reported world-wide as the causal agent of diseases of fruit trees, vegetables, cereals and ornamentals (reviewed in Schroth *et al.* 1992). Its widespread occurrence (and infection of a number of economically important crops) render it the subject of the majority of contemporary studies on different aspects of *P. syringae*-host plant interactions. The current status of different *P. syringae* pathovars in the international research arena was reflected by papers presented recently in Berlin, Germany (summarized in Table 2-1).

Table 2-1. Summary of *Pseudomonas syringae* pathovars and their host plants, as discussed in papers presented at the Fifth International Conference on *Pseudomonas syringae* pathovars and related pathogens, held in Berlin, Germany from 3-8 September 1995.

Pathovar	Host plant(s)	Author(s)
actinidiae	kiwi fruit	Janse, J. and Scortichini, M.
aptata	barley and wheat	Maraite, H. and Weyns, J.
apii	celery	Little, E. and Gilbertson, R.
atrofaciens	cereals (barley, wheat, rye, oats, triticale)	Al-Sallami, F., Karov, S.P., Vassilev, P., Popova, R. and Vassilev, V. Boukris, L. and Nassan, N.N. Iacobellis, N.S., Figiuolo, G., Janse, J.D., Scortichini, M. and Ciuffreda, G. Maraite, H. and Weyns, J. Pasichnik, L., Gvozdyak, R. and Chodos, S. Vassilev, V., Lavermicocca, P., Di Giorgio, D. and Iacobellis, N.S. Von Kietzell, J. and Rudolph, K.
coronofaciens	oats	Pasichnik, L., Gvozdyak, R. and Chodos, S.
coriandricola	coriander	Al-Shinawi, T. and Rudolph, K. Toben, H. and Rudolph, K.
fuscovagine	rice	Flamand, M.-C., Batoko, H., Boutry, M., Kinet, J.-M. and Maraite, H.
	wheat	Fucikovsky, L.
garcae	coffee	Kairu, G.M. Korobko, A. and Wondimagegene, E.
glycinea	soybean	Bogatzevska, N. Fucikovsky, L. Hutcheson, S.W., Pirhonen, M.U., Rowley, D.L., Lideli, M.C., Lee, S.W. and Keen, N.T. May, R., Völksch, B., Kampman, G. and Nüske, J. Müller, J., Völksch, B. and Fritsche, W. Weingart, H. and Völksch, B.
helianthi	sunflower	Fucikovsky, L.
lachrymans	cucumber	Collmer, A., Bauer, D.W., Alfano, J.A., Preston, G., Loneiello, A.O. and Milos, T.M.

Pathovar	Host plant(s)	Author(s)
lachrymans (continued)	squash	Fucikovsky, L.
malculicola	crucifers	Little, E. and Gilbertson, R.
marginalis	lettuce	Elumalai, R.P. and Mahadevan, A.
mori	mulberry	Baharuddin, U.P.
morspronorum	apricot and cherry	Wimalajeewa, S.
papulans	apple	Huang, T.C., Burr, T.J., Smith, C.A. and Matteson, M.C.
persicae	stone fruit	Vigouroux, A.
phaseolicola	bean	Adam, A.L. Bogatzevska, N. Fucikovsky, L. Hettwer, U., Nöllenburg, M., Koopmann, B. and Rudolph, K. Jansing, H. and Rudolph, K. Mansfield, J., Puri, N., Jenner, C., Stevens, C., Bennet, M.A., Tsiamis, G., Taylor, J. and Teverson, D. Panopoulos, N.J. Romantschuk, M., Roine, E. and Björklöf, K. Schaad, N.W., Hatziloukas, E. and Panopoulos, N.J. Weingart, H. and Völksch, B.
	kudzu	Weingart, H. and Völksch, B.
	yicama	Fucikovsky, L.
pisi	pea	Cournoyer, B. and Vivian, A. Durant, C.B. and Archer, S.A. Fucikovsky, L. Grondeau, C. and Samson, R. Jackson, R., Arnold, D., Cournoyer, B. and Vivian, A. Malandrin, L. and Samson, R. Roberts, S.J
savastanoi	oleander	Adinolfi, M., Corsaro, M.M., Evidente, A., Lanzetta, R., Marciano, C.E., Motta, A., Parrilli, M., Sonnenberg, B., Rudolph, K., Brusco, O. and Surico, G. Sisto, A., Morea, M., Zaccaro, F., Palumbo, G. and Iacobellis, N.S.

Pathovar	Host plant(s)	Author(s)
savastanoi (continued)	olive	Adinolfi, M., Corsaro, M.M., Evidente, A., Lanzetta, R., Marciano, C.E., Motta, A., Parrilli, M., Sonnenberg, B., Rudolph, K., Brusco, O. and Surico, G. Balestra, G.M. and Varvaro, L. Capasso, R., Christinzio, G., Evidente, A., Visca, C. and Iannini, C. Fucikovsky, L. Sisto, A., Morea, M., Zaccaro, F., Palumbo, G. and Iacobellis, N.S.
sesami	sesame	Prathuangwong, S. and Yowabhud, P. Fucikovsky, L.
syringae	apple	Bröther, H. Geider, K.
	apricot	Wimalajeewa, S.
	avocado	Fucikovsky, L.
	bean	Collmer, A., Bauer, D.W., Alfano, J.A., Preston, G., Loneiello, A.O. and Milos, T.M. Grgurina, I., Iacobellis, N.S., Camele, I. and Curci, R. Hatziloukas, E., Takikawa, Y., Schaad, N.W. and Panopoulos, N.J. Khan, I.D. and Rudolph, K. Lehman-Danzinger, H., Jarchow-Redecker, K. and Rudolph, K. Niepold, F. Romantschuk, M., Roine, E. and Björklöf, K.
	cherry	Gross, D.C., Hutschison, M.L., Scholz, B.K. and Zhang, J. Wimalajeewa, S.
	coriander	Fucikovsky, L.
	fruit trees	Zeller, W., Xie, Y., Bereswill, S. and Geider, K.
	kiwi fruit	Janse, J.D. and Scortichini, M.
	laurel	Di Giorgio, D., Camoni, L., Scaloni, A., Scortichini, M. and Ballio, A.
	mango	Cazorla, F.M., Olalla, L., Torés, J.A., Codina, J.C., Pérez-García, A. and De Vicente, A.
	nectarine	Appel, M., Mansvelt, E.L. and Bollstedt, D.U.
	parsley	Fucikovsky, L.
	pea	Kumar, J.K. and Nandita, P.
	pear	Bröther, H. Geider, K.
	pepper	Arsenijevic, M. and Obradovic, A. Hevesi, M. and Ledó, H.D.

Pathovar	Host plant(s)	Author(s)
syringae (continued)	Sudan grass	Yakovleva, L., Zdorovenko, G., Gvozdyak, R. and Solyanik, L.
	sugar beet	Maraite, H. and Weyns, J.
	wheat	Boukris, L. and Nassan, N.N.
tabaci	tobacco	Fucikovsky, L. Yakovleva, L.M., Pasichnik, L.A., Porembskaya, N.B., Zdorovenko, G.M. and Vassilev, V.I.
tagetis	tagetis	Fucikovsky, L.
tolaasii	champiñon	Fucikovsky, L.
	mushroom	Shirata, A. and Yoshida, S.
tomato	tomato	Bogatzevska, N. and Deneva, S. Fucikovsky, L. Habazar, T. and Rudolph, K. Little, E. and Gilbertson, R. Nabizadeh-Ardekani, F., Koopmann, B. and Rudolph, K. Pérez-García, A., Cánovas, F.M., Canón, F.J., Cazorla, F.M., Rivera, M.E. and De Vicente, A. Zdorovenko, G.M., Knirel, Y.A., Solyanik, L.P., Yakovleva, L.M. and Vassilev, V.I.

Papers were presented in seven sections, namely Ecology and Epidemiology, The resistant reaction of the plant, Pathogenesis, Determinants of pathogenicity, Genetic analysis of host/plant-interaction, Identification and Taxonomy and Control.

Each entry does not represent a separate paper, as more than one pathovar-host plant interaction were addressed by some authors.

In some of the molecular studies, specific pathovar-host plant interactions were not mentioned. These papers were omitted from the table.

A framework for understanding the genetic basis of host-specificity was established by Flor (1955, 1971, see also later elaborations by Ellingboe (1976) and Heath, 1991). His "gene-for-gene" hypothesis predicted that the specificity between a pathogen and a host plant essentially resided in the incompatible interaction and was genetically determined by concurrent expression of dominant "avirulence" (*avr*) genes in the pathogen and "disease resistance" genes in the host.

The validity of this hypothesis for bacterial pathogens was elegantly demonstrated by Staskawicz and co-workers (Staskawicz *et al.* 1984, 1985, 1987, Napoli and Staskawicz 1987, Keen and Staskawicz 1988, Tamaki *et al.* 1988, Keen *et al.* 1990), exploiting the phenomenon that certain cultivars of soybean specifically exhibit resistance against certain races of the soybean blight pathogen, *P. s.* pv. *glycinea*. Avirulence genes have been also been cloned (and gene products isolated and characterized in some cases) from other pathovars of *P. syringae*, such as pv. *phaseolicola* (Harper *et al.* 1988, Hitchin *et al.* 1989, Shintaku *et al.* 1989, Jenner *et al.* 1991), pv. *tomato* (Whalen *et al.* 1988, Kobyashi *et al.* 1989, Ronald *et al.* 1992, Innes *et al.* 1993, Salmeron and Staskawicz 1993, Lorang and Keen 1995), pv. *pisi* (Vivian *et al.* 1989) and pv. *maculicola* (Dong *et al.* 1991, Whalen *et al.* 1991). In the light of its atypically broad host-range, it is not surprising that none of these studies were conducted with *P. s.* pv. *syringae*. The molecular basis of the host-specificity (or apparent lack thereof) of this pathovar thus remains unclear. Although the study of *avr* genes is still pursued with vigour, the emphasis has shifted slightly towards elucidating the intriguing interaction between this gene system and *hrp* genes (see Salmeron and Staskawicz 1993, Hutcheson *et al.* 1995, Lorang and Keen 1995, Mansfield *et al.* 1995 for recent examples).

1.3. The nature and extent of diseases caused by Pseudomonas syringae

The majority of *P. syringae* pathovars cause necrotic diseases, which are manifested as blossom blasts, blights, leaf spots and cankers on stems and trunks (Roos *et al.* 1993). This is also true for *P. s.* pv. *syringae*. Due to the large and diverse host range of this pathovar, details of specific diseases will be limited to the description of bacterial canker caused by *P. s.* pv. *syringae* in stone fruit trees in South Africa, which follows later in this chapter.

The ability of *P. syringae* pathovars to cause these diseases in their respective hosts depends on a number of factors. These include environmental conditions, aspects of plant physiology (such as nutritional status and developmental stage) and the expression of pathogenicity and virulence factors by the bacterium. *Pathogenicity* refers to the genetic predisposition of a bacterium to be able to cause disease (Shaner *et al.* 1992). This fundamental ability to perturb the plasma membrane of

host cells, cause the leakage of water, cations and organic nutrients into the intracellular space where bacterial growth and multiplication is subsequently sustained, is controlled by *hrp* genes in phytopathogenic pseudomonads, xanthomonads and non-soft rot erwinias (Sigee 1993). Functions such as pathogen fitness and the ability to attach to the plant surface are also included in the broad definition of pathogenicity (Lamb *et al.* 1989). It is thus important to understand that, although the functional expression of *hrp* genes is required for pathogenicity, it alone is not sufficient for the development of disease (He *et al.* 1993).

All the strains of a particular bacterial pathovar will not affect the health of a host plant to the same extent. Strains capable of growing and spreading faster and causing more severe destruction of host tissue are said to be more *virulent* than others. Four major groups of extracellular molecules have been implicated in bacterial virulence, namely toxins, extracellular enzymes, extracellular polysaccharides (EPS) and plant hormones (Daniels *et al.* 1988, Shaner *et al.* 1992, Sigee 1993). The production, structure and function of these factors have been studied in a variety of bacteria (reviewed in Panopoulos and Peet 1985, Chatterjee and Vivader 1986, Sigee 1993). For the purpose of this study, a short summary of the general functions of these molecules and selected examples (see Table 2-2) will suffice.

According to Roos *et al.* (1993), most researchers regard *P. syringae* as a weak pathogen that causes disease only when its host is stressed. In this regard, it is considered an excellent opportunist. Its capabilities as such are attributed to three factors, namely:

(a) its ability to survive epiphytically on a number of plant surfaces (Ercolani *et al.* 1974, Lindow *et al.* 1988, Hirano and Upper 1990, Kinkel *et al.* 1995, Suoniemi *et al.* 1995) and latently inside the shoots, flowers and fruit of host plants (Roos *et al.* 1993);

(b) its wide host range (Schroth *et al.* 1992) and

Table 2-2. Summary of bacterial virulence factors.

Virulence factor	General function	Major associated disease type (causative pathogens)	Examples produced by *P. syringae* pathovars)	Selected references
Toxins	Non-enzymatic, chemical injury to host cells, resulting in the release of water and nutrients to create an environment in which the pathogen can migrate and grow	Necrotic diseases (pseudomonads, xanthomonads and non-soft rot erwinias	Coronatine, tagetitoxin, phaseolotoxin, syringomycin, syringotoxin, tolaasin, taboxin	Durbin (1983), Xu and Gross (1988), Bender *et al.* (1989), Rudolph (1990), Gross (1991), Rainey *et al.* (1991), Rich *et al.* (1992), Quigley and Gross (1994), Hutchison *et al.* (1995), Liyanage *et al.* (1995), Mo *et al.* (1995).
Extracellular polysaccharides	Induction and maintenance of water-soaking, prevention of bacterial desiccation and recognition by plant, occlusion of plant vessels. Also implicated in bacterial resistance to heavy metals and other toxic substances	Vascular diseases (Coryneform bacteria, some *Erwinia* spp., pseudomonads, xanthomonads and fastidious prokaryotes (e.g. *Xylella* spp.)	Levan, alignate	Bennet (1980), Fett *et al.* (1986,1989, 1992), Mansfield and Brown (1986), Dolph *et al.* (1988), Hignett (1988), Barta *et al.* (1992), Koa and Sequeira (1994), Rudolph *et al.* (1994), Hettwer *et al.* (1995), Kidambi *et al.* (1995).
Extracellular enzymes (pectolytic enzymes, cellulases, proteases)	Degradation of cell walls and other cellular components of the plant, utilization of these degradation products in the bacterial metabolism	Soft rot diseases (xanthomonads, pseudomonas and *Clavibacter* spp.)	Polygalacturonase	Collmer and Keen (1986), Allen *et al.* (1987), Wandersman *et al.* (1987) Boccara *et al.* (1988), Roberts *et al.* (1988), Tang *et al.* (1988), Pagel and Heitefuss (1990), Longland *et al.* (1992).
Plant hormone. (auxins and cytokinines)	Induction of the rate of plant cell division, leading to the formation of hyperplasia	Tumour diseases (*Agrobacterium* spp., *Pseudomonas syringae* pv. *savastanoi*)	Indole acetic acid (auxin), zeatin, zeatin riboside, 1'-methyl-zeatin, 1"-methyl-zeatin riboside (cytokinins)	Kosuge *et al.* (1966), Surico (1986), Da Costa E Silva and Kosuge (1991), Gardan *et al.* (1992b), White and Mazzola (1994).

(c) heterogeneity with regard to biochemical characteristics and the expression of virulence factors within populations on the same host (Endert and Ritchie 1984, Hirano and Upper 1990).

The abovementioned factors ensure that inoculum is available for dissemination when environmental conditions are suitable for infection (Hirano and Upper 1990).

Against this background, it is not surprising that reports on the occurrence of *P. s.* pv. *syringae* on different hosts in different regions of the world range from the description of latent infections (occurrence of the bacterium without any apparent disease symptoms) to the severe destruction of orchards and fields.

2. Bacterial canker of stone fruit in South Africa

Bacterial canker of stone fruit, caused by *P. s.* pv. *syringae*, has been reported from a variety of fruit producing countries in temperate and Mediterranean regions of the world (e.g. Crosse 1966, Latorre and Jones 1979, Roos and Hattingh 1986a, Paterson and Jones 1991, Wimjaleewa *et al.* 1991). In South Africa, the disease seems to be more severe than elsewhere in the world (Hattingh *et al.* 1989). For this reason, it has been researched extensively since the early eighties, mainly by Hattingh, Roos and Mansvelt (currently and/or formerly from the Department of Plant Pathology, University of Stellenbosch or the Agricultural Research Council's Institute for Fruit Technology (Infruitec) in Stellenbosch). The most important results of their studies are presented here.

2.1. Epidemiology

Bacterial canker of stone fruit has become one of the most destructive crop diseases in South Africa. Financial losses attributed to the disease are estimated to exceed R 25 million annually (Hattingh *et al.* 1989). Areas that are particularly hard hit are the South Western Cape, where apricot, plum, peach, nectarine, cherry and almond trees are affected, and the cherry-producing region of the Eastern Orange Free State (Roos and Hattingh 1983a, 1983b, 1986b).

Two pathovars of *P. syringae*, namely pv. *syringae* and pv. *morspronorum*, as well as intermediate forms linking the two pathovars, have been identified as the major

stone fruit attacking pathogens. *P. s.* pv. *morspronorum* was shown to be mainly restricted to the infection of cherries in the summer rainfall region (Orange Free State), whereas *P. s.* pv. *syringae* was found to be more widespread and general in the fruit-producing winter rainfall area of the Cape (Roos and Hattingh 1983a). Here, it has also been implicated in diseases of deciduous pome fruit, such as blister bark of apple (Mansvelt and Hattingh 1986a) and blossom blast of pear (Mansvelt and Hattingh 1986b).

In accordance with results from other studies (see earlier sections), the population of *P. s.* pv. *syringae* isolated from both diseased and symptomless stone fruit trees in South Africa were found to be heterogeneous with respect to their phenotypic characteristics (Roos and Hattingh 1983b, 1987a).

2.2. Symptoms and disease cycle

A number of symptoms are associated with bacterial canker of stone fruit trees (Fig. 2-1). The most conspicuous of these are gum-exuding branch and stem cankers, found frequently at bud unions of young trees and in pruning wounds on more mature trees (Roos and Hattingh 1983a, Hattingh *et al.* 1989). Cankered branches and stems die back within weeks, but the root system normally stays healthy and may develop suckers. Infection of dormant flower and leaf buds also occurs. Some of these buds are killed, others open in spring, but result in wilted leaves and dried out fruit. Others, still, will express no disease symptoms at all. Blossoms may also be infected. They will subsequently die, but typically remain attached to trees, and cankers will be formed on twigs and spurs (Hattingh *et al.* 1989).

Three factors contributed to the proposal of the life and disease cycle of bacterial canker caused by *P. s.* pv. *syringae* in stone fruit trees, illustrated in Fig. 2-2. They were:

(a) the isolation and observation (using scanning electron microscopy, SEM) of bacteria from symptomless organs of stone fruit trees (Roos and Hattingh 1983a, 1983b, 1986a, 1986b, 1987a and Hattingh *et al.* 1989), from poorly

germinating seeds (Hattingh *et al.* 1989) and from weeds growing in orchards (where they survive epiphytically) (Roos and Hattingh 1983a, 1986c);

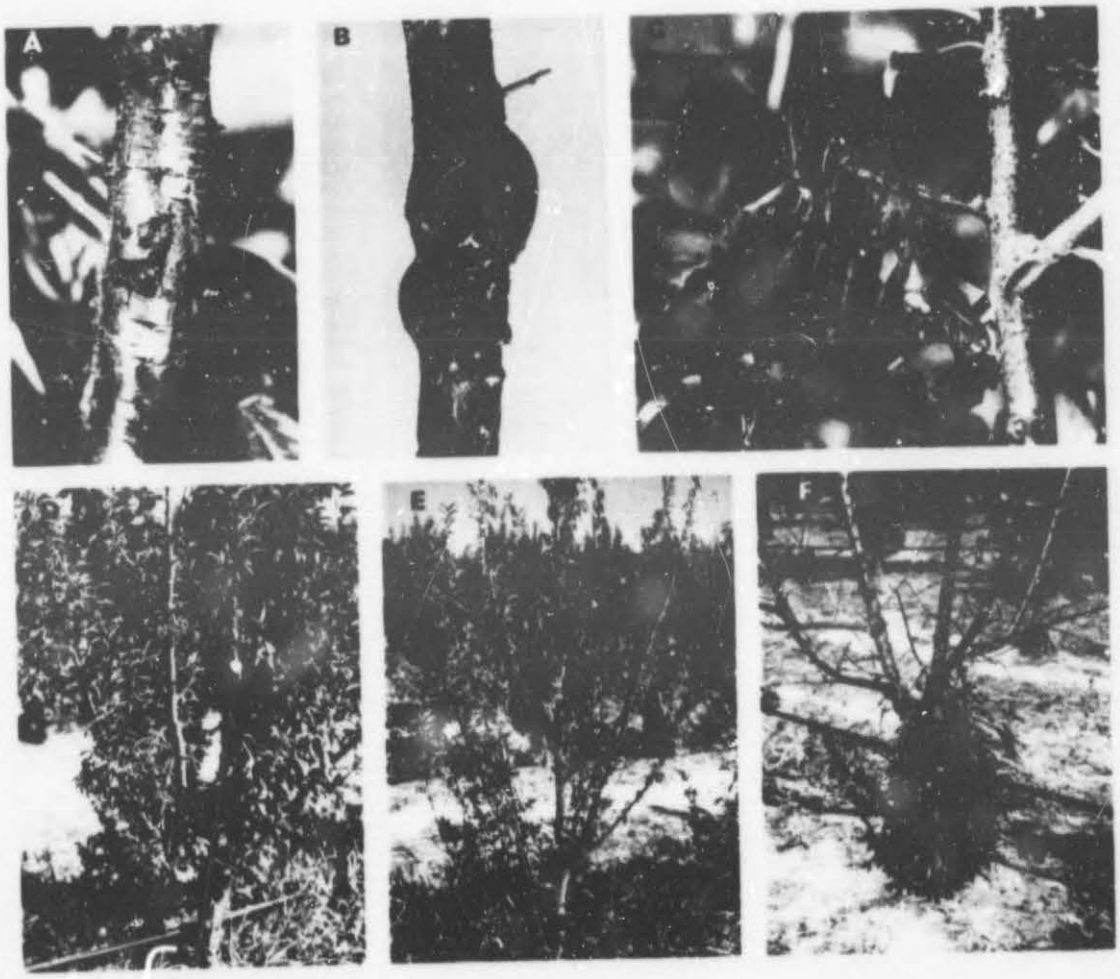

Fig. 2-1. Symptoms of bacterial canker of stone fruit trees, caused by *Pseudomonas syringae* pv. *syringae*.

A. Cankers on a peach trunk. **B.** Gum exuded from an apricot branch. **C.** Dieback of a twig on a plum tree during the middle of summer. **D.** and **E.** Cankered plum trees showing early and more advanced stages of dieback. **F.** Suckers developing from the rootstock of an otherwise dead tree. (Taken with permission from Hattingh *et al.* 1989).

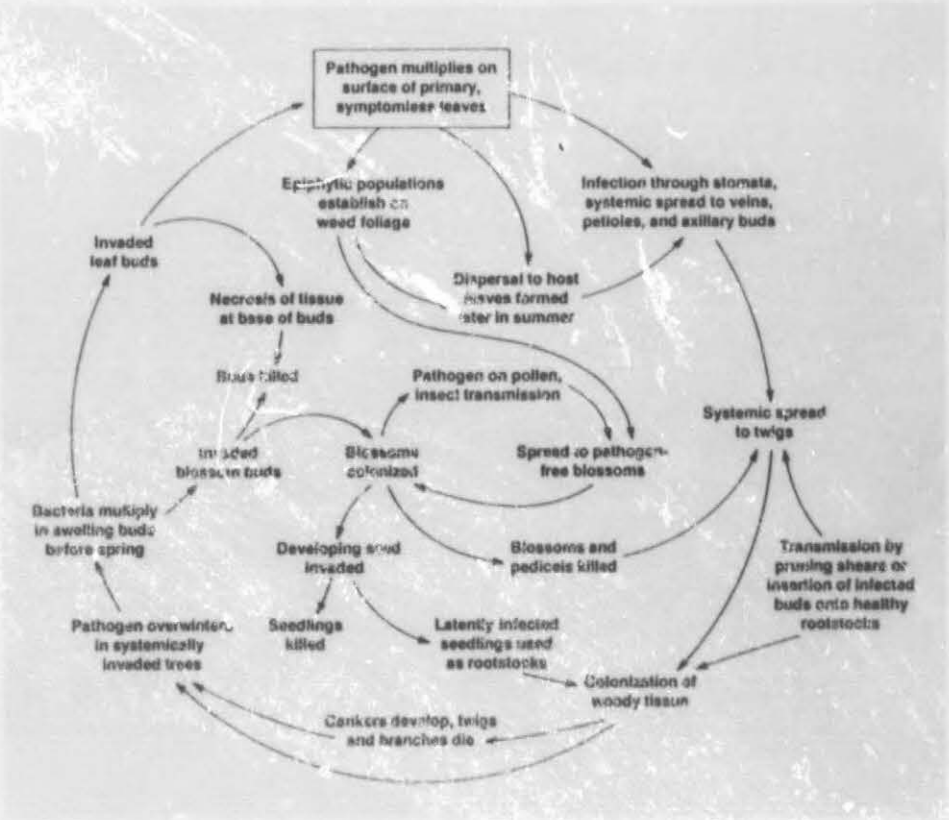

Fig. 2-2. Life and disease cycle of bacterial canker of stone fruit trees, caused by *Pseudomonas syringae* pv. *syringae*. (Taken with permission from Hattingh *et al.* 1989).

(b) studies (some employing SEM) showing systemic spread of bacteria in symptomless and infected trees (Roos and Hattingh 1987b, Hattingh *et al.* 1989, Roos *et al.* 1993) and

(c) the seasonal appearance of symptoms (disease activity is only noticed during spring and summer; Roos and Hattingh 1983a).

It must be noted that the disease cycle scheme is a generalized one, and that variations may occur in specific strain-cultivar interactions (Hattingh *et al.* 1989).

2.3. Control strategies

Reasons for the severity of bacterial canker of stone fruit trees in South Africa are not particularly clear. A combination of several predisposing factors have been suggested by Hattingh *et al.* (1989). These include climatic conditions (occasional droughts and mild winters, which do not meet the dormancy requirements of stone fruit trees) and stress-inducing horticultural practices (such as the establishment of orchards under marginal conditions, the application of copper sprays to achieve defoliation in the early winter and the introduction of the disease in nursery trees through the use of infected buds or rootstocks).

The development of an effective strategy to control the disease is thus still a high priority in South Africa. The heterogeneity of *P. s.* pv. *syringae* populations, together with the ability of the pathogen to survive and spread systemically in and on symptomless host plants, as well as on other, non-host plants, render this a daunting challenge. Furthermore, chemical control has failed completely in this country (Hattingh *et al.* 1989). For these reasons, it has been suggested that future efforts towards effective, integrated disease management will have to include the following essential elements (Hattingh *et al.* 1989, Roos *et al.* 1993):

(a) the improvement of horticultural practices known to favour disease development and aggravation;

(b) the control epiphytic population levels (see also Wimjaleewa *et al.* 1991) and

(c) the selection and breeding for disease resistance.

All three of these aspects are currently being addressed by Infruitec in the development of strategies for the control of bacterial canker in South Africa. High priority is placed on a resistance breeding programme. Progress in the application of classical selection and breeding techniques has, however, been limited, as none of the local commercially planted stone fruit cultivars exhibit natural resistance to *P. s.*

pv. *syringae*. As a result, investigations into the molecular basis of the host-pathogen interaction were initiated (E.L. Mansvelt, personal communication).

2.4. Molecular research approaches and local priorities

From the background given in the first part of this chapter it is evident that the interaction between *P. s.* pv. *syringae* and stone fruit trees is very complex. Nevertheless, three possible approaches towards the molecular study of this interaction were identified.

A first option would be to evaluate the involvement of virulence factors, such as toxins, in the severity of disease development. Although the experimental strain (NV) used in this study proved to be the most virulent of all locally isolated strains (E.L. Mansvelt, personal communication), an evaluation of virulence factors was considered of secondary importance in the design of effective control strategies. The main reason for this lies in the heterogeneity of the bacterial population occurring in natural inocula, as discussed earlier in this chapter.

A second possible approach would entail an investigation of the molecular basis of host-specificity, which resides in bacterial avirulence and plant resistance gene combinations. Such investigations currently enjoy much attention in the international research arena. The application of these studies in resistance breeding implies the identification of race-specific plant resistance genes which will result in incompatible combinations with corresponding pathogen avirulence genes, and may have to rely on the introduction of such genes in genetically manipulated cultivars (De Wit 1992). The atypical broad host range of *P. s.* pv. *syringae*, discussed earlier in this chapter, renders this approach an impractical one towards a solution for the South African situation.

The unique aspect of the South African bacterial canker scenario, outlined in the second part of this chapter, is that the disease is more destructive locally than elsewhere in the world. In the design of local molecular research projects in this field the emphasis must therefore be placed on the applicability of the study in the design of control strategies. This motivated Infruitec to follow the third possible approach, namely that of examining of the *hrp* gene cluster and/or product(s) of *P. s.* pv.

syringae NV, which control the fundamental ability of the bacterium to be pathogenic. The present investigation into the production of a harpin elicitor by *P. s.* pv. *syringae* NV was thus requested, with a view towards the possible application of a purified elicitor in a programme to screen local commercially important stone fruit cultivars for resistance against the bacterial canker pathogen on a biochemical basis.

The work presented in this thesis was facilitated by and based upon numerous recent studies on the unique *hrp* gene cluster and products of *P. s.* pv. *syringae* strain 61. The most important results of those studies will be presented in the next chapter.

CHAPTER 3

THE *HRP* GENES AND HARPIN ELICITOR OF *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE* STRAIN 61 AS MODELS FOR INVESTIGATIONS PRESENTED IN THIS STUDY

1. Introduction

The introduction of molecular biology techniques in studies on the hypersensitive response elicited by *Pseudomonas syringae* pv. *phaseolicola* (Anderson and Mills 1985, Lindgren *et al.* 1985, 1986, 1988, Somlayi *et al.* 1986, Deasy *et al.* 1987) and *P. s.* pv. *syringae* (Niepold *et al.* 1985, Panopoulos *et al.* 1985, Baker *et al.* 1987) led to the discovery of homologous gene clusters in these bacteria that controlled the development of both the HR on non-host plants and pathogenicity on host plants. The acronym *hrp* (for *h*ypersensitive *r*esponse and *p*athogenicity) was adopted for these gene clusters (Lindgren *et al.* 1986).

In subsequent studies, employing transposon mutagenesis, complementation analysis and DNA hybridization, *hrp* genes/gene clusters have been identified in other pathovars of *P. syringae*, such as pathovars *tomato* (Cuppels 1986, Bender *et al.* 1987), *pisi* (Malik *et al.* 1987), *angulata*, *tabaci* and *savastanoi* (Lindgren *et al.* 1988, Schroth *et al.* 1992), *glycinea* (Huynh *et al.* 1989), *morspronorum* (Liang and Jones 1995) and *aptata* (Minardi 1995b), as well as in other, distinctly different phytopathogenic species such as *Pseudomonas solanacearum* (Boucher *et al.* 1987, 1991, 1992, Huang *et al.* 1990, Arlat *et al.* 1992), *Erwinia amylovora* (Bauer and Beer 1987, 1991, Steinberger and Beer 1988, Barny *et al.* 1990, Walters *et al.* 1990) and pathovars of *Xanthomonas campestris* (Daniels *et al.* 1984, Kamoun and Kado 1990, Stall and Minsavage 1990, Arlat *et al.* 1991, Bonas *et al.* 1991, Waney *et al.* 1991, Schulte and Bonas 1992). Although total DNA homology between the *hrp* clusters of different species were found to be less pronounced than between that of different pathovars within a species, homologous loci have been identified in all of the known *hrp* clusters (Lindgren and Panopoulos 1986, Lindgren *et al.* 1988, Grimm *et al.* 1989, Roberts and Coleman 1991, Genin *et al.* 1992, Gough *et al.* 1992, Laby

and Beer 1992). Some of these loci proved to be involved in the regulation of (*hrp*) gene expression, or in the secretion of (*hrp* encoded?) proteins and in the regulation of secretion. Many valuable insights were gained from these studies, but a detailed description of the organization of each individual *hrp* gene cluster and discussions of the homology between the *hrp* genes and proteins of different species and pathovars falls beyond the scope of the present study. For this purpose, the reader is referred to reviews by Boucher *et al.* (1988), Beer *et al.* (1991), Bonas *et al.* (1991), Rahme *et al.* (1991), Willis *et al.* (1991), Arlat *et al.* (1991, 1992), Schroth *et al.* (1992), Van Gijsegem *et al.* (1993) and Liang and Jones (1995).

The complete functional *hrp* clusters of two pathogens, namely *Pseudomonas syringae* pv. *syringae* strain 61 (isolated from wheat by M. Sasser, but also shown to be a weak pathogen of bean; Atkinson and Baker 1987), and *Erwinia amylovora* strain Ea321 (causing fire blight of apple, pear and other rosaceous plants; Bauer and Beer 1991) have been isolated and cloned (Huang *et al.* 1988, Beer *et al.* 1991). These clusters were shown to be unique in their ability to enable non-pathogenic bacteria, such as *Pseudomonas fluorescens* and certain strains of *Escherichia coli* to elicit the HR in tobacco and other plants. Complementation of such bacteria with these clones was, however, not sufficient for the pathogenic phenotype (Huang *et al.* 1988, He *et al.* 1993), confirming the involvement of other genes (such as those required for pathogen fitness) in disease development on host plants (Panopoulos and Peet 1985, Lindgren *et al.* 1986).

Over-expression of subclones of the *hrp* clusters of *E. amylovora* Ea321 and *P. s.* pv. *syringae* 61 lead the discovery of the eternally elusive bacterial elicitors; the molecules which are directly responsible for HR elicitation (Wei *et al.* 1992a, He *et al.* 1993). Surprisingly enough, little homology was found between the genes encoding these proteins (named "harpins" by Wei and co-workers) or between the proteins themselves (He *et al.* 1993). The methodology used in these studies, however, paved the way for the isolation and characterization of the harpins of other phytopathogens, such as *P. s.* pv. *phaseolicola* (Panopoulos 1995).

The *hrp* gene cluster and particularly harpin$_{Pss}$ of *P. s.* pv. *syringae* 61 was used as a model for the present investigation. As such, the organization of the cluster and the

properties of individual genes/gene products, the regulation of gene expression, as well as the identification of *hrpZ2* and the characteristics and function of its protein product, harpin$_{Pss}$ will be discussed. The chapter will be concluded with remarks on the HR test for pathogenicity as a bioassay for elicitor activity.

2. The *hrp* gene cluster of *P. s.* pv. *syringae* 61

2.1. Organization and properties of individual genes/gene products

The 25 kilobase (kb) gene cluster isolated from *P. s.* pv. *syringae* 61 by Huang and co-workers (Fig. 3-1) was established in the cosmid pHIR11 (Huang *et al.* 1988). Transposon mutagenesis, complementation analysis and DNA sequencing led to the identification eight apparent transcriptional units (operons) in this cluster, six of which are polycistronic (Huang *et al.* 1991, Xiao *et al.* 1992, 1994, He *et al.* 1993, Hutcheson *et al.* 1994, Lidell and Hutcheson 1994). To date, twenty-four *hrp* genes (based on the phenotypic characteristics of *Tn5* mutants) have been identified (Lidell and Hutcheson 1994) and shown to be widely conserved among *P. syringae* pathovars and strains (Panopoulos *et al.* 1985, Lindgren *et al.* 1986,1988, Huang *et al.* 1991, Hutcheson *et al.* 1994). A twenty-fifth gene was shown to be unique to a subset of *P. syringae* strains, not required for pathogenicity and believed to be involved in the modulation of the HR, as *Tn5* mutants produced a delayed necrotic reaction in resistant hosts. This unit was subsequently designated *hrmA* (for *h*ypersensitive *r*eaction *m*odulation) (Huang *et al.* 1991, Heu and Hutcheson 1993, Hutcheson *et al.* 1994).

The biochemical functions of the majority of genes within the eight operons of the *hrp/hrm* cluster of *P. s.* pv. *syringae* 61 have not yet been established (Lidell and Hutcheson 1994). However, some of the loci have been characterized further, by determining their nucleotide sequences and deducing amino acid sequences and molecular masses of protein products resulting from the translation of open reading frames (ORF's) within these loci (see Fig. 3-1). Comparison of this data with the nucleotide and amino acid sequences of proteins with known functions revealed the following information:

(a) A region of *hrmA* product (41.5 kDa) exhibits homology with a virulence determinant (the *yopE* locus) of the human and mammal pathogen *Yersinia enterolitica*. The deduced nucleotide sequence of the complete gene product does, however, not reveal homology with any protein of known function and the biochemical function of hrmA is still unknown (Heu and Hutcheson 1993, Hutcheson *et al.* 1994, Xiao and Hutcheson 1994).

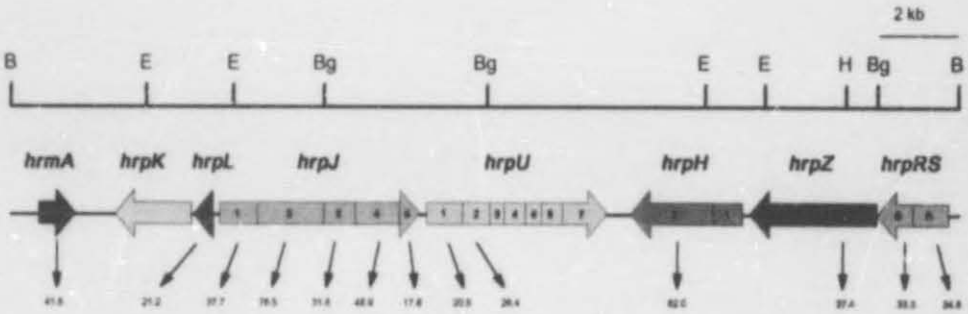

Fig. 3-1. Transcriptional and translational organization of the *hrp/hrm* gene cluster of *Pseudomonas syringae* pv. *syringae* 61 (Compiled from Huang *et al.* 1991, 1992, 1993, Xiao *et al.* 1992, 1994, He *et al.* 1993, Hutcheson *et al.* 1994, Lidell and Hutcheson 1994).

The eight transcriptional units (operons) and their orientations are indicated by the arrows. Designation of operons proceeded on the basis of homology (or lack thereof) with loci of the *hrp* clusters of other bacteria, particularly that of *P. s.* pv. *phaseolicola* (see Rahme *et al.* 1991). Open reading frames in polycistronic operons are numered sequentially from the 3'-end of each operon. The molecular masses in kilodalton (kDa) indicated for some of the gene products were deduced from the nucleotide sequences contained of ORF's and were confirmed by mass spectrometry and/or sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in some cases. Abbreviations for restriction sites are: B, *Bam*H1; Bg, *Bgl*I; E, *Eco*R1 and H, *Hind*III.

(b) *hrpK* is the only operon to have received little attention. To date, no biochemical function has been assigned to it or its product (Xiao *et al.* 1994, Xiao and Hutcheson 1994).

(c) The *hrpL* product (21.2 kDa) has been identified as an alternate sigma factor, homologous to AlgU of *Pseudomonas aeruginosa*. AlgU controls the expression of the *algD* operon, which encodes enzymes involved in extracellular polysaccharide biosynthesis (Martin *et al.* 1993, Xiao *et al.* 1994). The regulatory function of HrpL in the expression of other *hrp/hrmA* operons will be addressed in the next section.

(d) Five open reading frames have been identified in the *hrpJ* operon. The function of HrpJ1 (37.7 kDa) is unknown, but is believed to differ from the rest of the *hrpJ* gene products (Huang *et al.* 1993).

HrpJ2 (previously designated HrpI) has been identified a 76.5 kDa inner membrane protein (Huang *et al.* 1992, 1993) with characteristics similar to that of LcrD, a protein that was suggested to control the temperature and Ca^{2+}-dependent expression of several virulence proteins of the human pathogen *Yersinia pestis* (Plano *et al.* 1991). Other inner membrane proteins involved in protein secretion which share homology with *P. s.* pv. *syringae* 61 HrpJ2 are HrpI of *Erwinia amylovora* Ea321, involved in the secretion of harpin$_{Ea}$ (Wei and Beer 1993); VirH and InvA, involved in the secretion of invasion proteins by the human and animal pathogens *Salmonella flexneri* and *S. typhimurium* respectively (Galán *et al.* 1992); FlbF of *Caulobacter crescentus*, involved in the regulation of flagella expression (Ramakrishnan *et al.* 1991) and HrpO and HrpC2 of the plant pathogens *Pseudomonas solanacearum* (Gough *et al.* 1992, 1993) and *Xanthomonas campestris* pv. *vesicatoria* (Fenselau *et al.* 1992) respectively. The exact role of the latter two proteins have not yet been elucidated, but they were shown to be required for a HR^+ phenotype.

HrpJ3, (35.1 kDa) shows similarities with the FliG proteins of *Salmonella typhimurium* and *Bacillus subtilis*, whilst HrpJ5 (17.8 kDa) is homologous to the FliJ proteins of these bacteria. Both FliG and FliJ are associated with flagellar biosynthesis (McNab 1992, Lidell and Hutcheson 1994).

HrpJ4 (48.9 kDa) has been classified as a member of the FliI family of cytoplasmic ATPase-like proteins implicated in the provision of energy necessary for the translocation of (flagellar) proteins (Lidell and Hutcheson 1994). The family includes HrpB6 of *Xanthomonas campestris* (Fenselau *et al.* 1992), FliI of *S. typhimurium* and *B. subtilis* (Albertini *et al.* 1991, Vogler *et al.* 1991), Spa47 of *Shigella flexneri* (Venkatesan *et al.* 1992) and the β-subunit of the F_0F_1 -ATPase of *E. coli* (Saraste *et al.* 1981).

(e) The *hrpU* operon encodes seven genes. Of these loci, only *HrpU1* and *HrpU2* have been investigated. However, neither of the two deduced protein products (20.5 and 26.4 kDa respectively) exhibit similarity with any proteins of known function. Sequence motifs in *hrpU2* suggest that the deduced gene product may contain two possible transmembrane domains (Lidell and Hutcheson 1994).

(f) *hrpH* encodes a 82.0 kDa outer membrane protein, showing similarities with YscC of *Y. enterolitica*, PulD of *Klebsiella oxycota* (d'Enfert *et al.* 1989) and pIV of filamentous coliphages (Brisette and Russel 1990), all outer membrane proteins involved in protein or phage secretion (Michiels and Cornelis 1991, Michiels *et al.* 1991) (Huang *et al.* 1992).

(g) Six open reading frames have been identified in the *hrpZ* operon. The only charaterized locus, *hrpZ2* encodes the elicitor protein, $harpin_{Pss}$ (He *et al.* 1993). To date, this is the only known secreted *hrp* gene product. This protein will be discussed in more detail later in this chapter.

(h) The *hrpRS* operon is a homologue of *hrpRS* of *P. s.* pv. *phaseolicola*. Both gene products of this locus were classified as members of the NtrC family of two component regulatory proteins involved in the transcriptional activation and repression of many genes/operons in the enteric bacteria *Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella typhimurium* and the symbiotic plant bacteria *Rhizobium* spp., *Bradyrhizobium japonica* and *Agrobacterium tumefaciens*. A conserved DNA-binding carboxy-terminal domain is present in all of these proteins. HrpR (34.8 kDa) and HrpS (33.3 kDa) are, however, different from the other members of this family in that they lack an amino-terminal domain that modulates their regulatory activity (Grimm and Panopoulos 1989, Fellay *et*

al. 1991, North *et al.* 1993, Hutcheson *et al.* 1994, Xiao *et al.* 1994, Xiao and Hutcheson 1994, Grimm *et al.* 1995). Together with *hrpL*, *hrpRS* is implicated in the expression and the environmental regulation of expression of several *P. s.* pv. *syringae* 61 *hrp* loci, as discussed in the next section (Xiao *et al.* 1994, Xiao and Hutcheson 1994).

Characterized *P. s.* pv. *syringae* 61 *hrp/hrmA* gene products thus fall within three categories. Of these, the regulatory proteins (HrpL, HrpR and HrpS), which are involved in the regulation of *hrp/hrmA* gene expression and the plant response elicitor (HrpZ2 or haprin$_{Pss}$) are best characterized. The exact functions of the third group of proteins, showing homology with proteins involved in the secretion of virulence factors or flagella in other plant and animal pathogens (Hrp J2-J5, HrpU1 and U2 and HrpH) have not yet been elucidated, but it is believed that they form a distinct pathway for the export of pathogenicity proteins (such as harpin) in bacteria (Lidell and Hutcheson 1994). In this regard, mutations in the *hrpJ2*, *J4*, *J5*, *H* and *U2* loci have been shown to abolish the HR$^+$ phenotype (Huang *et al.* 1992, 1993, He *et al.* 1993, Lidell and Hutcheson 1994). The common ancestry and possible similar functions for homologous gene products of plant and animal pathogens implicated in such a unique secretory pathway has also been addressed by Gough *et al.* (1992), Van Gijsegem *et al.* (1993, 1994) and Fenselau *et al.* (1992).

In conclusion, it is interesting to note that a homologue of the *hrpM* locus, identified by Mukhopadhyay and co-workers (Mukhopadhyay *et al.* 1988) in *P. s.* pv. *syringae* strain PS9020 (the causal agent of brown spot on bean) has not been found in the *P. s.* pv. *syringae* 61 *hrp/hrm* gene cluster. A similar locus has, however, been identified and characterized in *P. s.* pv. *phaseolicola* (Mills and Mukhopadhyay 1990, Mindrinos *et al.* 1990). In both *P. s.* pv. *syringae* PS9020 and *P. s.* pv. *phaseolicola*, *hrpM* was shown not to be linked to the *hrp* clusters of these bacteria and was believed to be involved in the regulation of (*hrp*) gene expression or nitrogen uptake (Mukhopadhyay *et al.* 1988, Mills and Mukhopadhyay 1990, Mindrinos *et al.* 1990). High sequence homology between the *P. s.* pv. *syringae hrpM* locus and the *mdoGH* operon of *E. coli* was only shown. The latter is required for the synthesis of membrane-derived oligosaccharides found in the periplasmic space in response to low osmolyte environments (Loubens *et al.* 1993). If such an unlinked *hrp* locus

exists in *P. s.* pv. *syringae* 61, it is not required for conferring the HR$^+$ phenotype to non-pathogenic bacteria (Huang *et al.* 1991).

2.2. Regulation of hrp gene expression

An interesting aspect of the hypersensitive reaction induced by phytopathogenic bacteria in non-host plants is the so-called "induction period". Treatment of inoculated plants with antibiotics, which specifically inhibit bacterial RNA and protein synthesis, during this period prevents the development of HR symptoms. In the following "latent period", no symptoms are visible yet, but the development thereof cannot be abolished by antibiotic treatment (Klement and Goodman 1967a, 1967b; Sequeira 1975, Klement 1982, Sasser 1982). Molecular studies since the early nineties demonstrated this to be the period necessary for the irreversible induction of *hrp* gene expression and the synthesis of *hrp* gene products (Goodman and Novacky 1994). These studies also revealed the following aspects of the regulation of *hrp* gene expression in *P. s.* pv. *syringae* 61:

(a) Expression of the majority of *P. s.* pv. *syringae* 61 *hrp/hrm* operons increases after inoculation into a non-host plant. This increase in expression takes place within the induction period of the HR (Xiao *et al.* 1992). In agreement with the findings of Yucel *et al.* (1989), regulation of *hrp* gene expression during an incompatible reaction *in planta* was attributed to environmental factors, rather than to a plant effector, as suggested by Fellay *et al.* (1991) and Rahme *et al.* (1992) for the regulation of *hrp* gene expression in *P. s.* pv. *phaseolicola*. The possible role of plant signals in *hrp* gene regulation is still not yet clearly understood, as is indicated by later studies of Wei *et al.* (1992b) and Minardi (1995a) on the regulation of *Erwinia amylovora hrp* gene expression. The possibility that the *hrp* genes of *P. syringae* pathovars and *E. amylovora* are regulated via different mechanisms, has also been suggested by Xiao *et al.* (1992).

(b) Cultivation of bacteria on minimal salt media (limited in nitrogen sources and supplemented with selected carbon sources) results in enhanced expression ("derepression") of *hrp* genes (and a reduction in the induction period required

to initiate the HR). Nutritional regulation of *hrp* gene expression is also believed to take place *in planta* (Hutcheson *et al.* 1989, Xiao *et al.* 1992, He *et al.* 1993).

(c) At least one operon in the *P. s.* pv. *syringae* 61 *hrp/hrm* cluster is repressed by high osmolarity. Although similar results were reported for the expression of *P. s.* pv. *phaseolicola hrp* genes (Mindrinos *et al.* 1990), the significance of osmotic repression in the *in planta* situation is not yet clear (Xiao *et al.* 1992).

(d) Some *hrp* gene products have been implicated in the regulation of the expression of other genes within the *hrp/hrm* cluster. HrpL was shown to regulate the expression of *hrmA*, *hrpH*, *hrpJ*, *hrpK*, *hrpU*, *hrpZ*, as well as some avirulence genes. A conserved sequence motif exhibiting HrpL-dependent promoter activity has been identified in the promoter regions of the abovementioned *hrp* genes, as well as in the promoter regions of several avirulence genes (Xiao and Hutcheson 1994). The expression of HrpL is, in turn, regulated by HrpR and HrpS, which were shown to specifically activate the *hrpL* promoter. The regulatory sequences of *hrpL* recognized by HrpR and HrpS are believed to be located in the *hrpL-hrpJ* intergenic region (Xiao *et al.* 1994, Xiao and Hutcheson 1994).

(e) *hrp* gene expression and *avr* gene expression seems to be mutually dependent. The exact nature of this relationship has, however not been elucidated yet (Schroth *et al.* 1992, Hutcheson *et al.* 1993, 1995, Xiao and Hutcheson 1994, Collmer *et al.* 1995, Mansfield *et al.* 1995).

Similar results regarding the abovementioned four aspects have been obtained in studies on the regulation of *hrp* gene expression in *P. s.* pv. *glycinea* (Huynh *et al.* 1989), *P. s.* pv. *pisi* (Yucel *et al.* 1989), *P. s.* pv. *phaseolicola* (Lindgren *et al.* 1988, Staskawicz *et al.* 1988, Grimm and Panopoulos 1989, Grimm *et al.* 1989, Mindrinos *et al.* 1990, Rahme *et al.* 1991, 1992), *P. s.* pv. *tomato* (Salmeron and Staskawicz 1993, Shen and Keen 1993), *P. solanacearum* (Arlat *et al.* 1992, Genin *et al.* 1992, Gough *et al.* 1992) and *E. amylovora* (Wei *et al.* 1992b).

3. Harpin$_{Pss}$: elicitor of hypersensitivity/pathogenicity

3.1. Identification of hrpZ2

An outline of the approach employed by He *et al.* (1993) in the identification of the elicitor encoding gene (*hrpZ2*) of the *P. s.* pv. *syringae* 61 *hrp/hrm* cluster is given in Fig. 3-2, to provide a framework for understanding the approaches followed in the present study. Expression and purification of the recombinant elicitor protein product, harpin$_{Pss}$, is not included in this figure, as a very similar procedure was employed and will be described in detail later in this study.

Two HR eliciting proteins of different sizes (32 kDa and 42 kDa respectively, as predicted from SDS-PAGE) were found to be expressed by plasmids pSYH1 and pSYH4 indicated in the figure. Further subcloning revealed the 32 kDa protein to be a truncated derivative (lacking the N-terminal 125 amino acids) of the 42 kDa protein. The 42 kDa protein was named harpin$_{Pss}$ (to distinguish it from harpin$_{Ea}$ of *E. amylovora*, identified and characterized by Wei *et al.* 1992a) and proved to be the only HR elicitor encoded by the *hrp/hrm* gene cluster of *P. s.* pv. *syringae* 61. A subclone of plasmid pSYH4, named pSYH10, containing the complete ORF of *hrpZ2* (1026 bp), encoding harpin$_{Pss}$, was provided by He and co-workers for use in the present study. A partial restriction map of pSYH10, as well as the nucleotide sequence of *hrpZ2* will be given in a later chapter.

3.2. Characteristics

Harpin$_{Pss}$ was shown to be a 341 amino acid, extracellular protein with an exact molecular mass of 34.7 kDa (deduced from the amino acid sequence of the protein and confirmed by mass spectroscopy of the purified protein) (He *et al.* 1993). The same authors reported that harpin$_{Pss}$:

(a) is extremely sensitive to proteases;

(b) is heat-stable;

(c) is rich in glycine (13.5 %) and lacks cysteine and tyrosine;

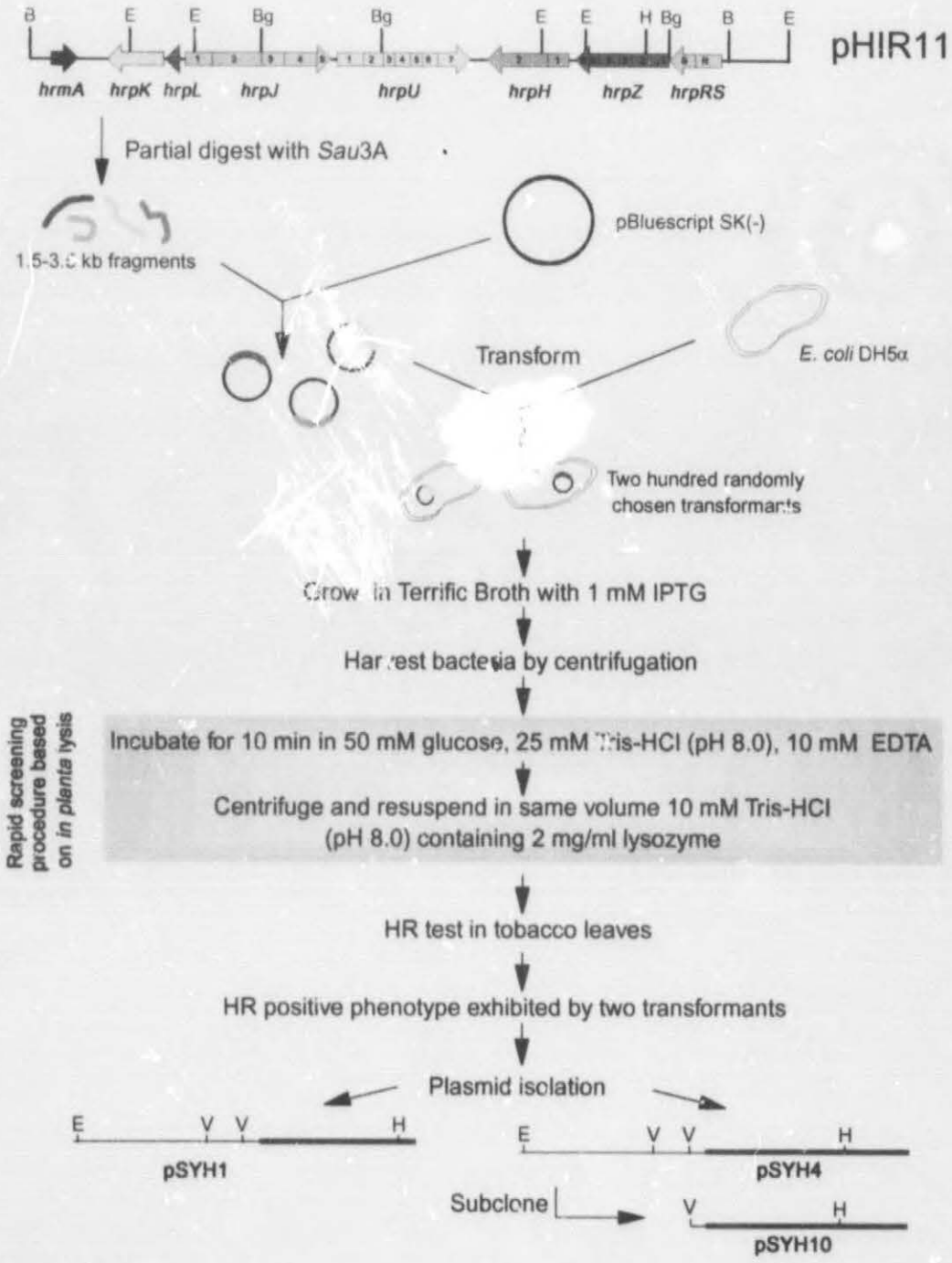

Fig. 3-2. Schematic representation of the methodology followed by He *et al.* (1993) in the identification of *hrpZ2*, the gene encoding the harpin elicitor of *Pseudomonas syringae* pv. *syringae* 61.

Abbreviations for restriction enzyme sites: B, *Bam*H1; Bg, *Bgl*I; E, *Eco*R1; H, *Hind*III; V, *Eco*RV.

(d) appears to be highly hydrophilic and is a soluble, cytoplasmic protein when expressed in *E. coli*;

(e) contains no amino-terminal signal peptide and is secreted in a *hrpH*-dependent manner;

(f) contains two repeated sequences, GGGLGTP and QTGT in its carboxy-terminal 148 amino acid portion (intact sequences of both repeats are necessary for elicitor activity);

(g) shows similarity with a region of the *E. amylovora* harpin over a stretch of 22 amino acids, of which eleven are identical in the two proteins (this homologous region is, however, not required for elicitor activity);

(h) share no significant sequence similarity with any protein of known function and does not contain any motifs of known biological significance;

(i) is only detected in the concentrated extracellular fluids of bacterial cultures grown in minimal media, indicating that the expression of this protein is under tight regulation and

(j) elicits the HR in plants (other than the normal hosts of *P. s.* pv. *syringae* 61), which results from an active response of the plant (involving *de novo* gene expression and protein synthesis, calcium flux across membranes and ATPase activity), rather than from a toxic effect. It, however, appears to be more effective in eliciting the HR when produced by living *hrp*$^+$ bacteria than when administered in its purified form.

3.3. Function

Despite being able to purify and characterize harpin$_{Pss}$, He and co-workers were not able to ascertain the exact role of this protein in pathogenesis. Being both necessary and sufficient for HR elicitation, it was suggested to be responsible for releasing nutrients from the plant cells into the apoplast. On the role of harpin$_{Pss}$ (and other harpins) in pathogenicity, they speculated further that, if harpin proved to be sufficient for pathogenicity, its identity or quantity should determine whether the

interaction between pathogen and plant leads to hypersensitivity or disease development (He *et al.* 1993).

It was also argued that it is likely that other factors influence the interaction between harpin and the plant cell. This theory was based on the fact that animal pathogens, such as *Yersinia*, secrete multiple virulence proteins through their secretion pathways indicated to be homologous to the bacterial *hrp* pathway. It was also suggested that other proteins secreted via the *hrp* pathway may have an effect on host specificity (He *et al.* 1993).

The key to a better understanding of the function of $harpin_{Pss}$ in pathogenicity is believed to lie in the as yet unidentified harpin receptor (predicted by Gabriel and Rolfe 1990) and current research efforts concentrate on this aspect (A. Collmer, personal communication).

4. The HR test for pathogenicity/elicitor activity

4.1. Historical aspects

The first description of incompatible (hypersensitive) reactions induced by phytopathogenic bacteria (Klement 1963) followed long after the first reports of hypersensitivity induced by phytopathogenic fungi (Ward 1902, Stakman 1915) and viruses (Holmes 1929). This was ascribed to the fact that the visible symptoms of bacterial-induced hypersensitivity are much less conspicuous in nature that those caused by fungi and viruses (Klement 1982). Despite the fact that as little as one bacterium can cause the death of one plant cell, the confluent necrosis typical of the incompatible interaction is only visible when 25 % or more of the plant cells in an affected region are killed (Turner and Novacky 1974). High numbers of incompatible bacteria (> 5×10^6/ml; Klement 1982, Lindgren and Panopoulos 1986, Grimm *et al.* 1989, Fett and Jones 1995), much higher than those present in natural inocula (Ercolani 1973, Rudolph 1975), are thus required for the development of a easily distinguishable HR. The injection-infiltration method developed by Klement and co-workers (Klement 1963, Klement *et al.* 1964) provided the means to introduce sufficiently high inocula of incompatible bacteria into plant tissues. As the induction

of the HR was shown to be a unique characteristic of pathogenic bacteria, a practical test for pathogenicity was established in this way (Klement 1982).

4.2. Practical considerations

The HR test for pathogenicity normally involves the infiltration of high levels (10^8 to 10^9 cells/ml) of actively growing bacteria into the intracellular spaces of a leaf, using a hypodermic syringe (with or without a needle) or spray infiltration (Sigee 1993). The injection method (first described by Klement, 1963) seems to be the more widely used. This technique has also become the standard bioassay to test for elicitor activity in bacterial fractions and protein preparations (Wei *et al.* 1992a, He *et al.* 1993, Hutcheson *et al.* 1994).

Theoretically, any organ of any plant which is a non-host or resistant host to the bacterium to be tested, can be used for this procedure. In practice, infiltrations are preferably done into the leaves of tobacco, pepper, tomato, bean, soybean and *Arabidopsis thaliana*. These plants are easy to grow and maintain under glasshouse conditions, yield clear HR symptoms with the majority of phytopathogenic bacteria and their fleshy leaves facilitates the infiltration procedure (Sigee 1993).

A typical HR is visually characterized by the appearance of a white, desiccated lesion over the entire infiltrated area within 8-24 hours (Hutcheson *et al.* 1989). However, a number of factors may influence the development of the HR, resulting in the appearance of less typical symptoms. These include environmental factors (e.g. temperature, light and humidity), the age of the inoculated leaf and the inoculum concentration (reviewed extensively in Klement 1982 and Sigee 1993). To avoid difficulties in the interpretation of HR test results, these variables should be kept as constant as possible. Infiltrations with bacterial fractions or purified protein preparations seem to elicit a less pronounced HR than that obtained with living bacteria. Although water-soaking of the infiltrated region appears within 24 hours, desiccation is delayed and the resulting lesion is more brown in colour (Wei *et al.* 1992a, He *et al.* 1993, this study). Typical HR phenotypes obtained with living bacteria and bacterial fractions are compared in Fig. 3-3.

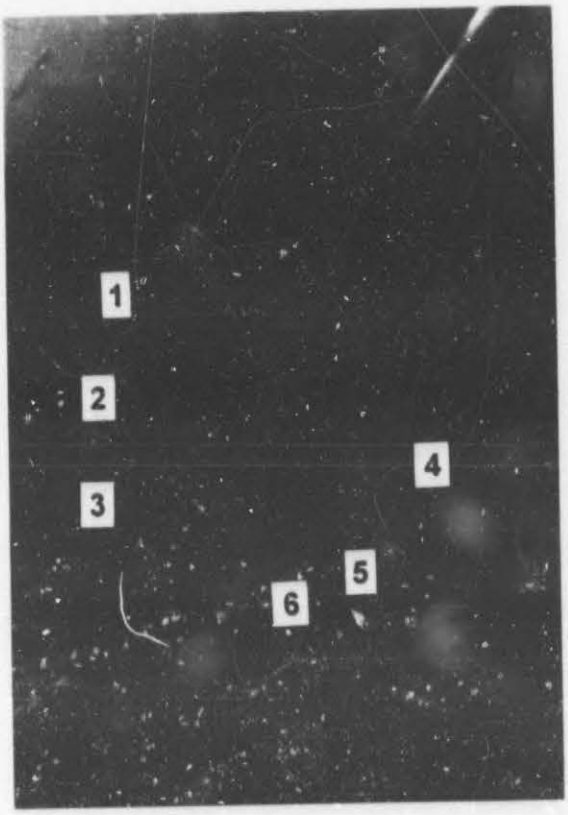

Fig. 3-3. Tobacco leaf showing typical HR phenotypes (48 hours after infiltration).

1: HR^+ phenotype when $> 10^8$ cells/ml of living bacteria (*P. s.* pv. *syringae* NV) was infiltrated, **2 and 3:** HR^+ phenotypes when infiltrations were done with elicitor containing fractions of *E. coli* DH5α (pSYH10), **4:** HR^- phenotype when *P. s.* pv. *syringae* NV was infiltrated at a concentration $< 10^8$ cells/ml, **5:** HR^- phenotype when a fraction of *E. coli* DH5α (pSYH10) that contain no elicitor was infiltrated, **6:** HR^- phenotype after infiltration with MES buffer (10 mM, pH 5.5).

Infiltrations were done as described in Materials and Methods (see following chapter). A number of chemicals were found to mimic an HR^+ phenotype when infiltrated above a certain concentration (e.g. PMSF > 0.1 mM; EDTA > 10 mM; NaCl > 0.5 M; NaN_3 > 0.1 % (m/v) and some culture media, such as the minimal media described in chapter 4). The observed effect depended on the nature and strength of the solution; some mimicked the typical HR^+ phenotype obtained with whole bacteria and others that obtained with bacterial fractions.

4.3. The ionic exchange response (XR)

One of the molecular characteristics during the early stages of the HR is the simultaneous influx of protons (H^+) into the plant cell (resulting in an increase in the extracellular pH) and the efflux of K^+ from the cell (leading to an increase in the extracellular conductivity) (Atkinson *et al.* 1985a, 1985b, Huang *et al.* 1988). The use of this "ionic exchange response" (XR) as a test for pathogenicity/elicitor activity, in conjunction with the use of suspensions of cultured tobacco cells, has yielded valuable insights into the molecular basis of the HR (Atkinson *et al.* 1985a, 1985b, 1990, 1993; Atkinson and Baker 1987, 1989; Baker *et al.* 1987, 1991, 1993, Keppler *et al.* 1989). However, the XR is logistically much more complicated than the HR and was therefore not used as a routine test for elicitor activity in this study.

5. Strategies followed in the current study

Despite the evidence presented in this chapter that the *hrp* genes are conserved in phytopathogenic pseudomonads and that homology exists between *hrp* genes/regions of these genes of different pathovars of *Pseudomonas syringae*, very little is still known about the harpins produced by different pathovars. For this reason it could not be accepted that the harpin produced by the locally isolated nectarine pathogen, *P. s.* pv. *syringae* NV, would be homologous to $harpin_{Pss}$ of the wheat pathogen to such an extent that the latter could be employed successfully in a stone fruit tree screening programme for resistance to bacterial canker. An investigation into the production of a harpin elicitor by *P. s.* pv. *syringae* NV was therefore deemed necessary by Infruitec.

With respect to the methodology to be used to this effect, two options were considered. The first was to follow the strategy employed by He *et al.* (1993), outlined in Fig. 3-2. (This approach was devised by Wei *et al.* (1992a) in the isolation of the first known harpin; that of the apple blight pathogen *Erwinia amylovora*.) A prerequisite to following this strategy was, however, that the *hrp* cluster of *P. s.* pv. *syringae* NV had to be identified and cloned.

Since a subclone of the cloned *hrp* gene cluster, pSYH10 (containing the whole open reading frame of the *hrpZ2* gene of *P. s.* pv. *syringae* 61), was made available

to us, an alternative strategy was decided upon. This would involve the transformation of *E. coli* DH5α with pSYH10, followed by expression, isolation and purification of the recombinant harpin$_{Pss}$. An anti-harpin$_{Pss}$ antiserum, raised in rabbit against the pure protein, would subsequently be used for the identification and purification (using an affinity chromatography system) of a possible harpin elicitor produced by *P. s.* pv. *syringae* NV. These experiments are described in the following chapter.

The availability of the *hrpZ2* gene also offered a simplified strategy for the location of the harpin encoding gene of *P. s.* pv. *syringae* NV. The polymerase chain reaction-based amplification of a *hrpZ2*-like DNA fragment from the *P. s.* pv. *syringae* NV genome, using primers based on the *hrpZ2* sequence, is described in chapter 5. Partial characterization of this fragment and subsequent arguments for its identity as the harpin encoding gene of the nectarine pathogen harpin are also presented.

CHAPTER 4

IDENTIFICATION OF A HARPIN ELICITOR PRODUCED BY *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE* ISOLATED FROM A NECTARINE TREE, USING ANTIBODIES AGAINST HARPIN$_{Pss}$ OF *P. S.* PV. *SYRINGAE* STRAIN 61

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ABSTRACT

The production of a harpin elicitor by *Pseudomonas syringae* pv. *syringae*, the causative agent of bacterial canker of stone fruit trees, has been investigated. Initial analyses of a South African strain (NV) that was isolated from a nectarine tree, indicated that the bacterium produces an elicitor of the hypersensitive response (HR) in tobacco. This elicitor exhibited characteristics similar to those of harpin$_{Pss}$, the harpin elicitor isolated previously from the wheat pathogen *P. s.* pv. *syringae* 61. A recombinant plasmid, pSYH10, containing the harpin$_{Pss}$ encoding *hrpZ2* gene of *P. s.* pv. *syringae* 61 (He *et al.* 1993) was used in this study to transform *E. coli* DH5α. The recombinant protein was subsequently expressed, purified and used to raise an anti-harpin$_{Pss}$ antiserum in rabbit. This antiserum was employed in Western blot analyses to identify a protein similar in size to harpin$_{Pss}$ in all elicitor active fractions and subfractions (obtained by ion exchange chromatography) of *P. s.* pv. *syringae* NV. The antiserum was, however, not specific and sensitive enough to facilitate the purification of this putative NV harpin elicitor from such fractions.

INTRODUCTION

Bacterial canker of stone fruit trees, caused by the pathogen *Pseudomonas syringae* pathovar *syringae*, has become one of the most destructive crop diseases in South Africa. Losses to the deciduous fruit industry are estimated to exceed US$ 10 million

annually. The severity of the disease, as well as the failure of chemical control in this country, has been attributed to a combination of climatic and soil factors and horticultural practices. An effective future control strategy will have to rely strongly on the breeding of resistant host trees (Hattingh *et al.* 1989). To assist in such breeding programmes, investigations into the molecular basis of the host-pathogen interaction were initiated.

Phytopathogenic bacteria are capable of eliciting a hypersensitive response (HR) in higher plants. The HR is characterized by a rapid, localized death of plant cells at the site of pathogenic invasion. It is typical of incompatible host-pathogen interactions (i.e. between a pathogen and a non-host or resistant host plant), is associated with resistance against pathogens and is used routinely as a test for pathogenicity (reviewed in Klement 1982, Sigee 1993, Goodman and Novacky 1994).

The ability of *P. syringae* strains to elicit the HR in non-host plants and to be pathogenic on host plants was shown to be controlled by *hrp* (*h*ypersensitive *r*eaction/*p*athogenicity) genes (Niepold *et al.* 1985, Lindgren *et al.* 1986, Willis *et al.* 1991). These genes are clustered, and some appear to be widely conserved in Gram-negative bacterial plant pathogens that cause eventual necrosis in their hosts, such as a number of *Pseudomonas*, *Xanthomonas* and *Erwinia* species and pathovars (He *et al.* 1993). The *P. syringae* molecule that actually elicits the HR, did however, stay elusive until 1993, when it was identified and characterized by He and co-workers (He *et al.* 1993). *P. s.* pv *syringae* 61 (a pathogen of wheat, also weakly pathogenic on bean; Atkinson and Baker 1987) was shown to produce a 34.7 kilodalton (kDa) extracellular protein elicitor of the HR, which was named harpin$_{Pss}$. This product of the *hrpZ2* gene of *P. s.* pv. *syringae* 61 was also reported to be heat-stable, sensitive to proteases, rich in glycine, produced only in apoplastic fluid-mimicking minimal media and secreted in a *hrpH*-dependent manner. The amino acid sequence of harpin$_{Pss}$ does not show similarity to that of any protein of known function. Although the exact function of harpin$_{Pss}$ in the elicitation of hypersensitivity or disease development has not yet been established, it has been shown to be necessary and sufficient for the HR$^+$ phenotype (He *et al.* 1993).

Against this background, we decided to investigate the production of a harpin elicitor by *P. s.* pv. *syringae* NV (a strain that was isolated locally from a nectarine tree). A subclone (pSYH10) of the cloned *hrp* gene cluster of *P. s.* pv. *syringae* 61, containing the whole open reading frame (ORF) of the harpin-encoding gene (*hrpZ2*), was made available for this study. It was therefore decided to attempt the identification and purification (using an affinity chromatography system) of a possible harpin elicitor produced by *P. s.* pv. *syringae* NV with the use of antibodies raised against the recombinant harpin$_{Pss}$. The success of this approach would depend on two factors, namely (a) that a highly specific and sensitive antiserum could be raised against harpin$_{Pss}$ and (b) that *P. s.* pv. *syringae* NV produces an elicitor sharing a reasonable degree of homology with harpin$_{Pss}$ on the protein level.

In this presentation, initial experiments on *P. s.* pv. *syringae* NV, aimed at establishing the characteristics and cellular location of a possible harpin elicitor produced by this bacterium, will be described first. This will include chromatographic procedures employed in the partial purification of such a protein from elicitor active fractions of the native bacterium. The expression and purification of a recombinant harpin$_{Pss}$, encoded by the *hrpZ2* ORF contained in pSYH10, and the production of anti-harpin$_{Pss}$ antisera will be described thereafter. The use of these antisera for the identification, but not for the purification, of a putative harpin elicitor of *P. s.* pv. *syringae* NV, will finally be presented.

MATERIALS AND METHODS

Cultivation of *P. s.* pv. *syringae* NV

P. s. pv. *syringae* NV (isolated from a nectarine tree, Roos and Hattingh 1987a) was obtained from the Infruitec (Institute for Fruit Technology, Stellenbosch, South Africa) culture collection. The bacterium was plated out from freezer stocks on King's B-agar (King *et al.* 1954) and grown for 16-24 hours at 27°C. It was subsequently used for the inoculation of growth cultures (250 ml each) in nutrient-yeast extract-glycerol broth (NYGB; 0.5 % (w/v) peptone, 0.5 % (w/v) yeast extract, 2 % (v/v) glycerol). Cultures were made in duplicate and incubated with constant shaking (225 rpm) at 27°C for 16-20 hours.

For ion exchange experiments (see later), *P. s.* pv. *syringae* NV was also grown in two different minimal media. The first was the minimal medium of Huynh *et al.* (1989) (50 mM phosphate buffer (pH 5.5), 7.6 mM $(NH_4)_2SO_4$, 1.7 mM NaCl, 1.7 mM $MgCl_2$), supplemented with 10 mM mannitol and either 10 mM D-fructose or 10 mM sucrose as carbon source. M9 minimal medium (Maniatis *et al.* 1989) (48 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 10 mM NH_4Cl, pH 5.7), supplemented with D-glucose, D-fructose or sucrose (2 % (w/v) respectively) as carbon source was also used for this purpose. Incubation conditions were the same as described above.

Determination of minimum inoculum density necessary for HR elicitation

The minimum inoculum density (colony forming units per ml, cfu/ml) of *P. s.* pv. *syringae* NV necessary to elicit the HR in tobacco was determined as follows. A fine suspension of *P. s.* pv. *syringae* NV from a King's B-agar plate culture was made in distilled water (9 ml). A dilution series (10^{-1} to 10^{-8}) of this suspension was prepared by five consecutive 1/10 dilutions in distilled water. Of each of the undiluted and diluted suspensions, 0.2 ml was infiltrated in duplicate in tobacco plants according to the procedure that is described below. The same quantity was plated out on King's B-agar in duplicate and incubated at 27°C. HR phenotypes, as well as the number of single colonies on each plate, were recorded 24 hours later. The inoculum density (cfu/ml) of each dilution was calculated, using average cfu values from duplicate plate counts.

Isolation of *P. s.* pv. *syringae* NV fractions

Bacterial cells were harvested from growth cultures by centrifugation (20 min at 9 000 x *g*). The culture supernatant (CSN) was decanted and stored at 4°C in the presence of 0.1 mM phenylmethylsulfonylfluoride (PMSF) for further treatment.

The bacterial pellet (cellular fraction) was washed three times in 0.1 M phosphate buffer (pH 6.5) and resuspended in 0.04 volumes of 10 mM (2-N-morpholino)-ethanesulfonic acid (MES; pH 5.5), supplemented with 0.1 mM PMSF. The suspension was subsequently sonicated on ice (Branson Model B-12 Cell Disruptor with microtip, 10 min at 50 % pulsed duty cycle) and centrifuged immediately

thereafter (27 200 x *g* for 15 min at 4°C). A small sample of the supernatant (sonication supernatant, SSN) was withdrawn, passed through a 0.22 µm filter and stored at 4°C in the presence of 0.1 mM PMSF. A sample (0.1 g) of the pellet (sonication pellet, SP) was resuspended in 0.5 ml MES (10 mM, pH 5.5, supplemented with 0.1 mM PMSF) and stored at 4°C. The remainder of the supernatant was maintained at 100°C for 10 min in a water bath and centrifuged (27 200 x *g* for 15 min at 4°C). The total final supernatant (boiled sonication supernatant, bSSN) and 0.1 g of the final pellet (boiled pellet, bP) were treated and stored as described for the SSN and SP above.

The cultura supernatant was filtered (through 0.45 µm and 0.22 µm filters consecutively) to remove residual bacteria. It was subsequently concentrated 10 to 50 fold by vacuum ultrafiltration, using an Immersible CX-10 filter (Millipore). The resultant concentrated culture supernatant (cCSN) was stored at 4°C in the presence of 0.1 mM PMSF and NaN_3 (0.1 % w/v). An aliquot of this preparation was maintained at 100°C for 10 min and centrifuged as described before, to obtain the boiled concentrated culture supernatant (bcCSN).

Protein determinations

The total protein content of bacterial fractions solutions were determined using the method described by Bradford (1976). The method was modified slightly to allow the simultaneous determination of multiple samples. Of each final reaction (50 µl protein suspension incubated for 2-30 min with 2.5 ml Bradford reagent), 200 µl were pipetted in duplicate into a flat-bottomed 96-well microtiter plate and absorbances (A_{620})[1] read at 620 nm with a microtiter plate reader (Multiskan Plus, Titertek). Bovine serum albumin (fraction V, Boehringer Mannheim; 1 mg/ml in 10 mM MES (pH 5.5) containing 0.1 mM PMSF), was used for the determination of a standard curve (A_{620} *vs* protein content, sensitivity range: 0-1 mg/ml). Bacterial fractions with total protein content exceeding 1 mg/ml were diluted in the same MES buffer for accurate

[1] In the original protocol absorbances of protein-dye complexes were measured at 595 nm (Bradford 1976). Spectrophotometric determinations have, however, shown the absorbance maximum of these complexes to be close to 620 nm (results not shown). The standard 620 nm filter of the microtiter plate reader could therefore be used effectively in Bradford protein determinations.

determination. The total protein content of each undiluted sample was calculated from standard curves by on-line computer.

Detection of elicitor activity by HR tests in tobacco

The HR phenotypes of bacterial fractions were determined by infiltration into tobacco leaves. Tobacco plants (cultivar White Burley) of uniform height (60-70 cm) were used and infiltrations were usually done in the late afternoon. An aliquot (0.2 ml) of each sample was infiltrated into a separate leaf section with a needleless syringe. All infiltrations were done in duplicate (using two different plants) and HR phenotypes were recorded 48 hours after infiltration. As controls for cellular fractions and subfractions, the buffers in which these fractions were maintained were infiltrated at their working concentrations. Uninoculated culture media were used as controls for culture supernatants. Samples of which the corresponding buffer or uninoculated medium elicited a HR-like reaction were dialyzed overnight at 4°C against 100-500 volumes of MES (10 mM, pH 5.5, containing 0.1 mM PMSF), after which the HR test was repeated.

SDS-PAGE of bacterial fractions

Protein profiles of bacterial fractions were compared by separation on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). An aliquot of each sample to be analyzed was mixed with an equal volume of treatment buffer (0.125 M Tris-Cl, 4 % (w/v) SDS, 20 % (v/v) glycerol, 10 % (v/v) 2-mercapto-ethanol, pH 6.8) and 0.2 volumes of bromophenol blue (0.1 % (w/v) in a 1.5 mM NaOH solution), and maintained in a boiling water bath for 2 minutes. Electrophoresis was carried out at 15 mA in a 7 cm x 8 cm x 1.5 mm resolving gel (12 % T, 2.7 % C_{bis}, 0.1 % SDS, pH 8.8), using a Hoefer SE 200 Mighty Small™ Vertical Gel Electrophoresis Unit. Eight- or ten-well stacking gels (3 % T, 2.7 % C_{bis}, 0.1 % SDS, pH 6.8) and a single tank buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS, pH 8.3) were used in this system. Rainbow™ coloured protein molecular weight markers (Amersham) were treated in the same manner as bacterial fractions for use as protein standards (14.3-220 kDa).

Protein bands were visualized after staining of the resolving gel (6 hours in 0.025 % (w/v) Coomassie Blue R-250, 40 % (v/v) methanol), followed by destaining (6 hours in Destain 1, 50 % (v/v) methanol, 10 % (v/v) acetic acid, and overnight in Destain 2, 7 % (v/v) acetic acid, 5 % (v/v) methanol). When results were required more rapidly, staining (2 hours) and destaining (2 hours in Destain 1 and 4 hours in Destain 2) were carried out at 37°C. The molecular weight (M_r) of protein bands of interest were calculated by comparison of their relative mobilities (R_f values) with those of the protein standards, plotted on a calibration curve (R_f values *vs* log M_r). The R_f value of a particular band was obtained by division of its mobility (mm from top of resolving gel) by the corresponding mobility of the dye front.

Separation of *P. s.* pv. *syringae* NV fractions by ion exchange chromatography

Two factors were taken into consideration in the choice of the elicitor active *P. s.* pv. *syringae* NV fraction to be used as starting material for further fractionation (i.e. partial purification of the elicitor protein) by ion exchange chromatography. These were the complexity of the protein profile and the elicitor content of the fraction. The bSSN seemed to be the least complex of all elicitor containing fractions and was thus used in initial analyses. However, according to He *et al.* (1993), more than 50 % of $harpin_{Pss}$ was secreted into the culture supernatant. This fraction could be obtained more quickly and in larger quantities than the bSSN, rendering it more suitable for repeated analyses. Culture supernatants were subsequently prepared from both NYGB and minimal media growth cultures of *P. s.* pv. *syringae* NV and subjected to resolution by ion exchange chromatography.

All separations were carried out on a DEAE-Sepharose CL-6B (weak anion exchanger, Pharmacia) column (length, 23 cm; internal diameter, 1.9 cm; bed height, 17.5 cm; bed volume, 50 cm^3), at flow rates of 1-2 ml/min. Fractions were collected at five minute intervals and their protein content monitored at 280 nm using a MSE Spectroplus detector.

Separation of bSSN

The *P. s.* pv. *syringae* NV bSSN was dialyzed overnight at 4°C against 800 volumes of starting buffer (25 mM Tris(hydroxymethyl)-aminomethane (Tris), pH 7.3, supplemented with 0.1 mM PMSF). It was subsequently resolved in this buffer (Tris A), using a linear salt gradient (0-1 M NaCl) for the elution of column-bound components. The complete salt gradient consisted of one bed volume of Tris A, followed by six bed volumes of Tris A and Tris B (mixed to form a linear gradient) and finally, two bed volumes of Tris B (Tris A plus 1 M NaCl). Individual peaks were identified from the chromatogram. Their corresponding fractions were pooled and concentrated by vacuum ultrafiltration for subsequent HR testing and SDS-PAGE, using procedures as described above.

A secondary separation was conducted on the pooled, concentrated fractions of peaks exhibiting elicitor activity. They were dialyzed against starting buffer (10 mM MES, pH 6.3, plus 0.1 mM PMSF) as described above. The salt gradient employed in this secondary separation consisted of one bed volume of the abovementioned MES starting buffer (MES A), followed by six bed volumes of MES A and MES B (MES A plus 1 M NaCl), mixed to form a linear salt gradient. The elution was concluded with two bed volumes of MES B. The fractions of each of the secondary peaks were again pooled, concentrated by vacuum ultrafiltration, tested for elicitor activity and subjected to SDS-PAGE.

Separation of cCSN

A similar salt gradient in MES buffer was used for the resolution of the cCSN obtained from *P. s.* pv. *syringae* NV grown on NYGB. It differed from the abovementioned profile only in the second phase. A shallower salt gradient (0.25-1 M NaCl, formed by mixing of 60 ml MES A and 150 ml MES B) was used for the elution of column-bound components in this instance. The sample was obtained in starting buffer conditions by five consecutive 1/10 dilutions with MES buffer A, each followed by concentration to the original sample volume. A substantial amount of inactive, yellow-coloured, low molecular weight medium components were excluded by means of this process. Peaks were identified, treated and analyzed as described before.

A variety of salt gradients were used in efforts to obtain better resolution of the NYGB cCSN or elicitor active peaks thereof. Different buffers (10 mM MES, pH 6.3; 10 mM Tris, pH 7.1; 25 mM Tris, pH 7.1 and 10 mM phosphate, pH 6.3) were employed. The length (six to nine bed volumes), form (linear, non-linear, continuous, discontinuous and combinations thereof) and range of the salt gradient (0-0.1 M NaCl, 0-0.2 M NaCl and 0-1 M NaCl and combinations thereof) were also varied. A number of these separations were also performed on culture supernatants from *P. s.* pv. *syringae* NV grown on minimal media. Full details of each separation will not be given here, but will be addressed where results are shown.

Preparation and transformation of competent *E. coli*

Plasmid pSYH10 (containing the *hrpZ2* ORF of *P. s.* pv. *syringae* 61) was obtained from Alan Collmer, Cornell University, Ithaca, New York. Frozen stocks of competent cells of *E. coli* strain DH5α were prepared and transformed according to the method described by Maniatis *et al.* (1989), using 1 ng of pSYH10 plasmid DNA per 50 µl aliquot of frozen competent cells. As controls for the experiment, one aliquot of competent cells was transformed with 1 ng pBluescript SK(-) DNA (Stratagene; this was the phagemid vector[2] used to construct plasmid pSYH10) and another aliquot with an equivalent volume (1 µl) of sterile distilled water. Transformed cells were plated out on SOB-agar medium (2 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 8.5 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 1.5 % (w/v) agar), supplemented with 20 mM $MgSO_4$ and 40 µg/ml ampicillin, and grown at 37°C for 16 hours. Individual colonies were each transferred aseptically to 10 ml Luria-Bertani (LB) medium (1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.17 M NaCl) containing 40 µg/ml ampicillin and grown overnight (16 hours) at 37°C in a shaking incubator (225 rpm). Freezer stocks of each of these overnight cultures (1 ml aliquots, containing a final concentration of 40 % (v/v) glycerol) were made and stored at -80°C. Clones were labelled alphabetically using the prefix pSYH10-. Clone pSYH10-a was used in all

[2]Phagemid vectors are plasmids containing an origin of replication derived from a filamentous bacteriophage (This allows cloned foreign DNA segments to be obtained in single-stranded form for sequencing purposes). The propagation and isolation of double-stranded DNA from foreign segments cloned in such plasmids, as carried out in this study, proceed in the conventional way (Maniatis *et al.* 1989). No further distinction will therefore be made between plasmids and phagemids.

further manipulations. Control clones (containing the unmodified pBluescript SK(-) vector) were labelled alphabetically using the prefix pBS-.

Evaluation of successful transformants by restriction digest of isolated plasmid DNA

To verify that successful transformants contained the correct plasmids, overnight cultures were made of clone pSYH10-a and control clone pBS-a, as described above (± 10 µl freezer stock in 10 ml LB medium containing 40 µg/ml ampicillin for each clone). Bacterial cells (2 x 3 ml per clone) were pelleted by centrifugation and plasmid DNA extracted according to the manufacturer's instructions, using a Plasmix Miniprep kit (Talent). The quantity and purity of isolated DNA (diluted 1/12) was assessed from absorbance readings at 260 nm (A_{260}) and 280 nm (A_{280}), using a Beckmann DU 650 spectrophotometer with mini (60 µl) cuvette. Undiluted DNA concentrations were calculated using the following conversion factor: A_{260} = 1 = 50 µg/ml double stranded DNA (Maniatis *et al.* 1989).

Restriction enzyme digestion of the isolated plasmid DNA (1 µg from each clone) was carried out by incubation with *Hind*III (Boehringer Mannheim, 10 units) in SuRE cut Buffer B (Boehringer Mannheim) at 37°C for 1 hour. Restriction digest reaction products, as well as aliquots of uncut isolated plasmid DNA, were each mixed with 0.2 volumes of gel loading buffer (15 % Ficoll (Type 400, Pharmacia), 0.25 % bromophenol blue, 0.25 % xylene cyanol FF) and separated on a 7 cm x 10 cm x 5 mm 0.75 % agarose gel (SeaKem LE agarose, FMC) in 1 x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) using a HE 33 Hoefer Minnie™ Horizontal Agarose Submarine electrophoresis unit. Ethidium bromide (0.33 µg/ml) was included in the gel for visualization of the electrophoresis result under ultra-violet (UV) illumination. Fragments generated by *Pst*1 digestion of λ-DNA (1 µg) were used as molecular mass markers. The estimated molecular mass (M_r) in base pairs (bp) of DNA fragments of interest were calculated by comparison of their relative mobilities (R_f values) with those of the DNA standards, plotted on a calibration curve (R_f values *vs* log M_r).

Expression of recombinant harpin$_{Pss}$, isolation and characterization of bacterial fractions

Harpin$_{Pss}$, the elicitor of *P. s.* pv. *syringae* 61, encoded by the 1026 bp *hrpZ2* ORF contained in plasmid pSYH10, was expressed essentially as described by He *et al.* (1993). An overnight culture of pSYH10-a was made in LB medium (containing 40 µg/ml ampicillin) as described before. Of this culture 5 ml was used for the inoculation of a growth culture in 250 ml Terrific Broth (Tartof and Hobbs 1987), containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 40 µg/ml ampicillin. The bacterium was subsequently grown at 30°C for 16-20 hours with constant shaking at 225 rpm. All cultures were made in duplicate and the protocol was carried out in parallel with the control clone pBS-a.

Bacteria were harvested from grown cultures by centrifugation (20 min at 9 800 x *g*), washed three times in 0.1 M phosphate buffer (pH 6.5), resuspended in 0.04 volumes of 10 mM MES (pH 5.5, supplemented with 0.1 mM PMSF) and sonicated as described before. All cellular fractions (SSN, SP, bSSN and bP) were collected, treated and stored as described for isolation of *P. s.* pv. *syringae* NV fractions. Culture supernatants were not collected, as the recombinant bacterium does not possess the ability to export harpin$_{Pss}$ out of the cell (He *et al.* 1993).

Protein determinations, HR tests for elicitor activity and SDS-PAGE were conducted with aliquots of all four fractions of clone pSYH10-a and the control clone pBS-a, according to previously described procedures.

Purification of recombinant harpin$_{Pss}$

Two different approaches were followed to purify the harpin$_{Pss}$ protein to homogeneity from the partially purified, heat-treated bSSN fraction of pSYH10-a.

Purification by non-denaturing PAGE

The first approach involved the separation of the bSSN on vertical non-denaturing polyacrylamide gel electrophoresis (ND-PAGE), followed by elution of the harpin$_{Pss}$ band from excised gel slices. Electrophoresis was carried out in a discontinuous

system, using a Hoefer SE 600 Dual Cooled Vertical Slab Gel Electrophoresis Unit and different tank buffers (Upper tank buffer: 37.6 mM Tris, 40 mM glycine, pH 8.89; Lower tank buffer: 63 mM Tris, 0.5 N HCl, pH 7.47). An aliquot of bSSN (4 ml; total protein content, 12.8 mg) was mixed with 0.1 volume of sucrose-dye solution (50 % (w/v) sucrose, 0.1 % (w/v) bromophenol blue in a 1.5 mM NaOH solution) and loaded in a single-well stacking gel (3 % T, 20 % C_{bis}, pH 6.9) for separation in a 14 cm x 16 cm x 1.5 mm resolving gel (12 % T, 5 % C_{bis}, pH 8.48).

Upon completion of electrophoresis, three strips (1 cm wide) were excised from the middle and two ends of the resolving gel. The position of the recombinant $harpin_{Pss}$ band was determined by fixation of these gel strips (1 hour in 12.5 % trichloroacetic acid, TCA), followed by staining (1 hour in 0.25 % Coomassie Blue G-250) and destaining (2 hours in 7 % (v/v) acetic acid, 5 % (v/v) methanol). The horizontal section of the resolving gel (± 16 cm x 2 cm) containing the recombinant protein was subsequently cut out, chopped finely and the protein eluted, using a Biometra Elucon apparatus. Elution was carried out for 48 hours at 20 mA in half-strength ND-PAGE upper tank buffer, supplemented with 0.1 mM PMSF. The eluted protein was extracted in eleven aliquots (100 µl each) according to the manufacturer's instructions and pooled. The total protein content was assayed by the Bradford method and the purified protein preparation was analyzed on SDS-PAGE as described previously.

Purification by ProSieve agarose gel electrophoresis

The ProSieve™ Gel System (FMC) was used in the second approach to purify the recombinant $harpin_{Pss}$. According to the manufacturer, native proteins can be separated in this gel system, formulated from derivatized agaroses, in a manner similar to an 8-15 % gradient polyacrylamide gel. The system also provides for easy, non-electrophoretic recovery of specific protein bands from the gel.

A vertical 16 cm x 14 cm x 1.5 mm ProSieve resolving gel with a single-well stacking gel was cast according to the manufacturer's instructions, using a Hoefer SE 600 Dual Cooled Vertical Slab Gel Electrophoresis Unit. An aliquot of pSYH10-a bSSN (5 ml; total protein content, 12 mg) was mixed with sucrose-dye solution as described before and electrophoresed using a 10 x Laemmli electrode buffer (0.25 M

Tris, 1.92 M glycine, pH 8.3) as anodal buffer and a standard (1 x) Laemmli buffer as cathodal buffer (Laemmli 1970).

Upon completion of electrophoresis, the position of the recombinant harpin$_{Pss}$ band was determined as described for the previous purification experiment. However, due to the different nature of the ProSieve gel, no TCA fixation was performed. Different staining (0.5 % (w/v) Coomassie Blue R-250 in 4 % methanol, 10 % acetic acid) and destaining (20 % methanol, 5 % acetic acid) solutions were also used. The relevant gel section was subsequently cut out and the harpin$_{Pss}$ protein recovered according to the manufacturer's instructions, using the abovementioned ND-PAGE lower tank buffer (supplemented with 0.1 mM PMSF) as extraction buffer. The protein content of the resulting protein suspension was assayed by the Bradford method and its purity analyzed on SDS-PAGE as described before. The remainder was lyophilized and stored at 4°C.

Production of rabbit anti-harpin$_{Pss}$ antisera

Polyclonal antisera was raised against the recombinant harpin$_{Pss}$ using the method described by Bellstedt *et al.* (1987). A solution of the lyophilized protein (40 µg/ml), as well as a fine suspension of lyophilized acid-treated *Salmonella minnesota* R595 bacteria (so-called naked bacteria, NB; 2 mg/ml), was made in double distilled water. The two components were added in a mass ratio (protein : NB) of 1 : 5. The protein antigen was absorbed to the surface of naked bacteria (immune carrier) by drying of the mixture on a rotary evaporator and resuspension in phosphate buffered saline (PBS; pH 7.2) to a concentration of 40 µg protein/ 0.5 ml suspension. This protein-NB complex is stable at 4°C for up to two weeks. An antigen suspension was therefore prepared freshly every 14 days for the duration of the immunization schedule, using the same lyophilized ProSieve gel purified harpin$_{Pss}$.

A rabbit was immunized by intravenous injection of the antigen suspension (0.5 ml) according to a fixed immunization schedule (on days 1, 4, 7, 11, 15, 18, 21, 25, 32, 35 and 39), designed for maximal antibody production with this method. Small blood volumes were drawn on days 0 (before immunization; control serum), 11, 18, 25, 32 and 39 and a final large volume on day 46. The blood was allowed to clot (1 hour at

37°C), after which the antiserum was isolated by centrifugation (10 min at 800 x *g*) and stored at -20°C.

Western blot analyses of *E. coli* DH5α (pSYR10) and *P. s.* pv. *syringae* NV protein fractions using anti-harpin$_{Pss}$ antisera

Western blot analyses were carried out essentially as described by Engelbrecht (1994). Prior to immunoblotting, all samples were subjected to 12 % SDS-PAGE, as described before. Protein profiles were subsequently transferred electrophoretically (16-20 hours at 120 mA) to a nitrocellulose membrane (0.45 µm, Schleicher and Schuell) in a buffer consisting of 0.05 M Tris, 0.2 M glycine and 20 % (v/v) methanol (pH 8.3). The Western blot analysis was performed in a multi-step procedure, all but the last step being carried out under constant agitation at 37°C. Firstly, the unoccupied area of the nitrocellulose membrane was blocked with casein buffer (154 mM NaCl, 0.5 % (w/v) casein, 10 mM Tris-Cl, 0.02 % thiomersal, pH 7.6) for 30 min. This was followed by incubation with the desired dilution (in casein buffer) of the primary antibody (rabbit anti-harpin$_{Pss}$ antiserum) for 2 hours. Excess antibody was removed by five washes of 4 min each in PBS-Tween (0.15 M PBS, pH 7.2, supplemented with 0.1 % (v/v) Tween-20). The membrane was subsequently incubated with sheep anti-rabbit antibodies (1/400 dilution in casein buffer) for 90 min. After repetition of the washing step, the membrane was incubated with rabbit peroxidase-anti-peroxidase complex (Sigma, diluted 1/5 000 in casein buffer) for 90 min and washed again.

Specific protein bands were visualized as black/blue precipitates by addition of substrate solution (4-chloro-1-naphtol (18 mg) dissolved in cold methanol (6 ml), which was added to 30 ml PBS (pH 7.2) containing 9 µl of a 34 % H_2O_2 solution). The substrate reaction was terminated after 30 min at room temperature by washing the membrane three times with distilled water. Due to the light-sensitivity of substrate reaction products, membranes were blotted dry and stored in aluminium foil.

Evaluation of antiserum specificity and sensitivity

The specificity and sensitivity (i.e. levels of specific antibodies) of the anti-harpin$_{Pss}$ antisera was evaluated in the following experiments. A constant quantity of

E. coli DH5α (pSYH10) bSSN was electrophoresed in different lanes on a 12 % SDS gel, as described before. Rainbow molecular weight markers were loaded in one or two other lanes, as required. The electrophoretic pattern of two lanes of the gel (one with bSSN and another with marker proteins) were visualized by Coomassie staining as described before. After electro-transfer of the proteins in remaining lanes, the nitrocellulose membrane was cut into strips, each corresponding to a lane of bSSN. A lane of Rainbow protein standards were included in one of these strips.

Western blot analysis was performed identically on all strips according to the above procedure, except that a different dilution of the anti-harpin$_{Pss}$ antiserum (e.g. 1/100, 1/500, 1/1 000 and 1/5 000) was used on each strip. The procedure was performed for antisera collected on days 18, 32 and 46 of the immunization schedule. These sera are indicative of the development of the immune response to the first (days 1, 4, 7 and 11), second (days 18, 21 and 25) and third (days 32, 35 and 38) rounds of antigenic challenge respectively. A progressive increase in the sensitivity and specificity of the antiserum for its antigen is expected. In order to assist in the interpretation of results, aliquots of the lyophilized, recombinant ProSieve gel purified harpin$_{Pss}$ used for the preparation of immunization complexes, were included in the Western blot analysis of the day 32 antiserum.

Western blot analysis of P. s. *pv.* syringae *NV fractions*

The fractions analyzed in this experiment differed slightly from those obtained by the routine fractionation protocol described previously. An aliquot of the total *P. s.* pv. *syringae* NV sonicate (before centrifugation, thus containing all soluble and insoluble proteins) was run instead of the sonication pellet. The SSN (total soluble proteins) and bSSN (total heat-stable soluble proteins) were standardly prepared. Five fold concentrations of the bSSN were made by rotary evaporation and lyophilization respectively. The cCSN was obtained and concentrated (10 fold and 30 fold) as described previously. The HR phenotype of each fraction was determined using the described procedure.

These *P. s.* pv. *syringae* NV fractions were separated on 12 % SDS-PAGE in duplicate as described previously. Also included in this separation were Rainbow protein standards and an aliquot of *E. coli* DH5α (pSYH10) bSSN, from which the

recombinant harpin$_{Pss}$ was purified for antibody production. One of the SDS-PAGE gels was subjected to Coomassie staining and the other to electro-transfer and Western blot analysis. A 1/500 dilution of the day 18 anti-harpin$_{Pss}$ antiserum was used as primary antibody. Aliquots of the *P. s.* pv. *syringae* NV cCSN (30 fold concentrated) and the *E. coli* DH5α (pSYH10) bSSN were run on a separate gel and analyzed with a 1/500 dilution of the day 0 (control) antiserum in order to identify bands resulting from the amplification of non-specific antibody interactions. The estimated sizes of specific bands recognized by the antiserum were determined from the Rainbow marker calibration curve for the specific separation as described previously.

Western blot analysis of P. s. *pv.* syringae *NV fractions resolved by ion exchange chromatography*

Subfractions of the *P. s.* pv. *syringae* NV bSSN and cCSN, obtained by ion exchange chromatography, as well as aliquots of unresolved bSSN and cCSN, were separated on 12 % SDS-PAGE and subjected to electro-transfer and Western blot analysis as described above. Day 18 anti-harpin$_{Pss}$ antiserum (1/500 dilution) was used as primary antibody solution. The experiment was carried out in duplicate, using a 1/500 diluted day 18 rabbit anti-lysozyme antiserum (prepared in the same way as the anti-harpin$_{Pss}$ antiserum) as primary antibody. This antiserum was chosen for use as a control, as it would give an indication of "non-specific" antibody interactions resulting from antibody clones in the anti-harpin antiserum that were raised against antigenic determinants on the immune carrier (naked bacteria). This would provide a better control than the day 0 anti-harpin$_{Pss}$ antiserum used previously, for reasons that will be discussed later (see Results of the evaluation of antiserum specificity and sensitivity).

RESULTS

Cultivation of *P. s.* pv. *syringae* NV on different media

Growth of *P. s.* pv. *syringae* NV proceeded much better on NYGB than on different minimal media, as was to be expected. The best growth on a Huynh minimal medium was obtained when mannitol and sucrose were added as carbon sources (57 % of

growth on NYGB, on the basis of wet cell mass). However, when M9 medium was supplemented with sucrose, only 33 % of growth on NYGB medium was achieved. The following growth yields were obtained on other minimal media (expressed as % of the growth on NYGB, on the basis of wet cell mass): Huynh medium supplemented with mannitol and D-fructose, 50 %; M9 medium supplemented with D-glucose, 41 % and M9 medium supplemented with D-fructose, 45 %.

Minimum *P. s.* pv. *syringae* NV inoculation density required for HR elicitation

The results of this experiment are given in Table 4-1. The HR was only elicited by the undiluted starting suspension and a 1/10 dilution thereof. An inoculation density exceeding 8.5×10^6 cfu/ml is thus required for elicitation of the HR in tobacco by *P. s.* pv. *syringae* NV. This result corresponded with the figure of $> 5 \times 10^6$ cfu/ml, typically quoted as the requirement for HR induction in non-host plants by phytopathogenic bacteria (Klement 1982, Lindgren and Panopoulos 1986, Grimm *et al.* 1989, Fett and Jones 1995).

Table 4-1. Determination of the minimum inoculation density (cfu/ml) of *P. s.* pv. *syringae* NV required for HR elicitation in tobacco.

Dilution of starting suspension	Number of cfu in 0.2 ml (average of 2 plate counts)	Inoculation density (cfu/ml)	HR
10^0	NC*	8.5×10^8	+
10^{-1}	NC*	8.5×10^7	+
10^{-2}	NC*	8.5×10^6	-
10^{-3}	NC*	8.5×10^5	-
10^{-4}	NC*	8.5×10^4	-
10^{-5}	1.7×10^3	8.5×10^3	-

* Not countable. The number of colonies in 0.2 ml of these dilutions were too many to count. The inoculation densities of such solutions were derived from calculations, using the number of countable colonies in higher dilutions and the appropriate dilution factor.

Elicitor activity and protein profiles of *P. s.* pv. *syringae* NV fractions

A number of important conclusions regarding the characteristics of the elicitor produced by *P. s.* pv. *syringae* NV grown on NYGB could be drawn from results of HR tests, which are given in Table 4-2. All of these characteristics were similar to those reported for harpin$_{Pss}$ (He *et al.* 1993).

Table 4-2. Evidence regarding the characteristics of the *P. s.* pv. *syringae* NV elicitor derived from by HR phenotypes of bacterial fractions.

Bacterial fraction	HR phenotype	Conclusion
Sonication supernatant (SSN)	+	Elicitor is water soluble
Sonication pellet (SP)	-	Elicitor is water soluble
Boiled sonication supernatant (bSSN)	+	Elicitor is heat-stable
Boiled pellet (bP)	-	Elicitor is heat-stable
Culture supernatant (CSN)	-	Elicitor is produced in small quantities
Concentrated culture supernatant (cCSN)	+	Elicitor is produced extracellularly
Boiled concentrated culture supernatant (bcCSN)	+	Elicitor is heat stable

The other important characteristic reported for harpin$_{Pss}$, namely its sensitivity to proteases (He *et al.* 1993), was also confirmed for the *P. s.* pv. *syringae* NV elicitor by HR test results. When bacterial fractions were not stored at 4°C in the presence

of PMSF, elicitor activity was lost within a few hours. The working concentration of PMSF was lowered to 0.1 mM (as used by Wei *et al.* 1992a) throughout this study, (compared to 1 mM used previously; He *et al.* 1993). This seemed to be effective in preserving elicitor activity, without causing HR-like symptoms on its own, as was found for 1 mM solutions of this protease inhibitor.

Protein profiles of the different *P. s.* pv. *syringae* NV fractions, as obtained by 12 % SDS-PAGE are shown in Fig. 4-1. Hoat treatment of the cCSN did not result in any significant changes to its protein profile, therefore this step was omitted from the routine fractionation protocol.

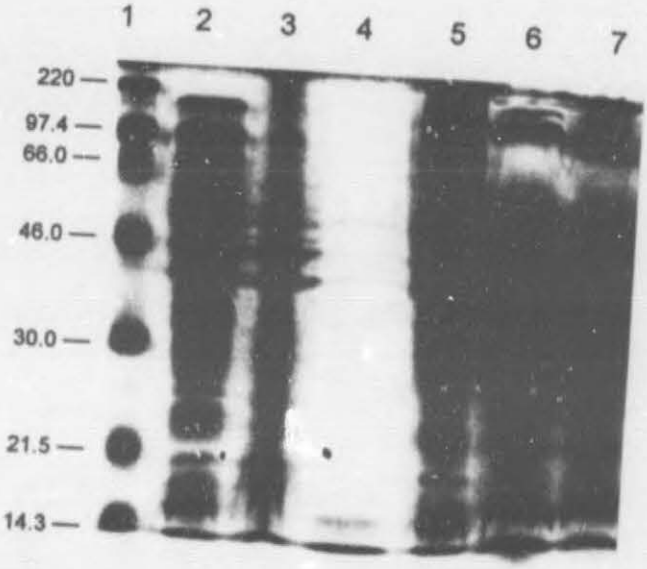

Fig. 4-1. Protein profiles of bacterial fractions from *P. s.* pv. *syringae* NV, as obtained by 12 % SDS-PAGE.

Lane 1: Rainbow marker proteins standards, **lane 2:** SSN (29 µg), **lane 3:** SP, **lane 4:** bSSN (4.8 µg), **lane 5:** bP, **lane 6:** cCSN (20 x concentrated, 58 µg), **lane 7:** bcCSN (57 µg).

SDS-PAGE was carried out as described in Materials and Methods. The total amount of protein loaded in each lane, determined with the Bradford method, is given in parenthesis. Protein concentrations for pellet fractions could not be determined accurately, as resuspended pellets did not form homogenous suspensions. The sizes (kDa) of protein standards are given on the left. An aliquot of the CSN was not included in this experiment. The protein concentration of this fraction was very low (0.21 mg/ml) and virtually no bands could be seen on SDS-PAGE.

The HR test results did not provide any clues on the expected size of the *P. s.* pv. *syringae* NV elicitor. Protein profiles of fractions shown to contain the elicitor (SSN, bSSN and cCSN) were compared in the hope of finding a band (or bands) of the same size occurring in all of these fractions (the possibility that the elicitor could be modified during export from the cell was not excluded, but such modifications were reported by He *et al.* (1993) not to take place during the export of harpin$_{Pss}$).

Despite the fact that relatively few bands were present in the bSSN, no conclusive evidence could be obtained in this way. This was mainly attributed to the apparent lability of the *P. s.* pv. *syringae* NV elicitor and indicated that it was not being produced in high quantities.

Results obtained from cultivation on minimal media

The HR was induced by all concentrated culture supernatants of *P. s.* pv. *syringae* NV grown on minimal media. However, each of the corresponding uninoculated control media produced the same reaction. Infiltration of cCSN preparations after dialysis yielded a negative result in every instance, leading to the conclusion that the initial positive results were artefacts caused by components in the culture media. Protein concentrations in the cCSN preparations were also low (0-0.1 mg/ml) and virtually no bands could be seen on SDS-PAGE of these preparations (not shown).

Resolution of *P. s.* pv. *syringae* NV elicitor active fractions by ion exchange chromatography

Resolution of bSSN

The chromatogram representing the separation of the *P. s.* pv. *syringae* NV bSSN is given in Fig. 4-2 A. A total of seven peaks (P1-P7) were identified. No proper resolution was, however, obtained between P4 and P5, and a degree of overlapping of these peaks occurred with P6.

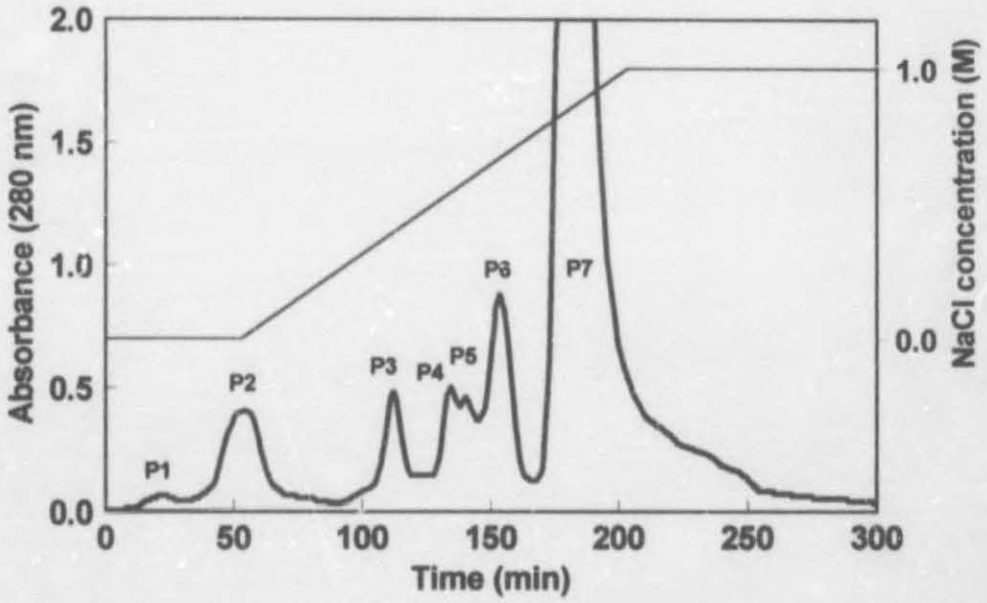

Fig. 4-2 A. Resolution of the *P. s.* pv. *syringae* NV bSSN by ion exchange chromatography.

Chromatography was carried out as described in Materials and Methods. The total protein content of the bSSN loaded on the column, as determined by the Bradford method, was 2.3 mg. The salt gradient (indicated in blue) is expressed in terms of the progressive addition of salt to the starting buffer (25 mM Tris, pH 7.3, supplemented with 0.1 mM PMSF) for the elution of column-bound components. Fractions were collected at five minute intervals. Fractions corresponding to the peaks indicated were pooled for further analysis.

After concentration (20 to 25 fold), HR activity was only exhibited by P4, P5 and P6. No conclusive evidence as to the identity of the elicitor could, however, be obtained from the protein profiles of these peaks (Fig. 4-2 B). The profiles were very complex and similar to each other (due to poor resolution) and also to the unresolved bSSN.

This led to the decision to perform secondary separations under different chromatographic conditions. The fractions of P4 and P5 were pooled (P4&5) for this purpose, but P6 was kept separate. The secondary separation of P4&5, conducted at a lower pH (to achieve earlier elution of weakly anionic column-bound components), yielded two large peaks (Fig. 4-3 A).

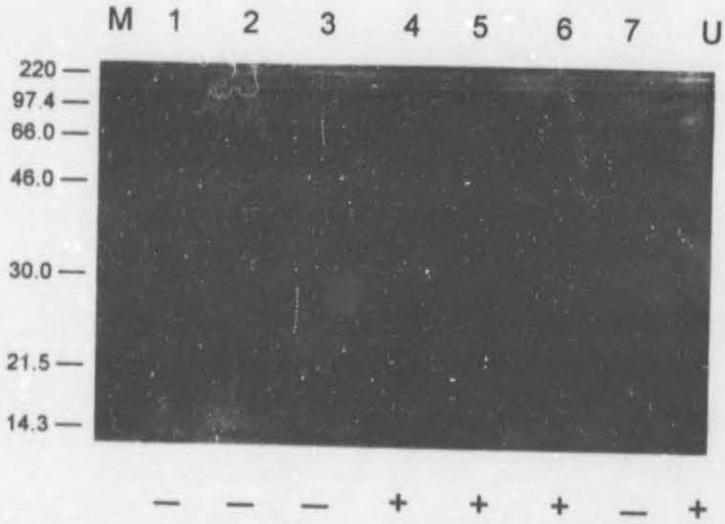

Fig. 4-2 B. Protein profiles on 12 % SDS-PAGE of peaks obtained by ion exchange chromatography of the *P. s.* pv. *syringae* NV bSSN.

Lane M: Rainbow protein standards. **Lanes 1-7** correspond with P1-P7 in Fig. 4-2 A. **Lane U:** unresolved bSSN (5.3 µg).

SDS-PAGE and protein determinations were carried out as described in Materials and Methods. The total protein content in aliquots of concentrated peaks loaded on the gel were: P1, 1.0 µg; P2, 0.13 µg; P3, 0.25 µg; P4, 7.5 µg; P5, 5.4 µg; P6, 2.6 µg; P7, 0.88 µg. The size (kDa) of protein standards are given on the left. The HR phenotype of each protein preparation is indicated at the bottom of each lane.

Subsequent analyses revealed only the weaker anionic one of the two (Q1) to be HR positive. Protein profiles of Q1 and Q2, obtained by 12 % SDS-PAGE (Fig. 4-3 B) differed significantly. Q1 exhibited a very complex protein composition. It was similar to the patterns observed for P4-P6 in the first separation, as well as with that of the unresolved bSSN. This corresponded with HR test results; yet no conclusive evidence on the identity of the *P. s.* pv. *syringae* NV elicitor was gained from these experiments.

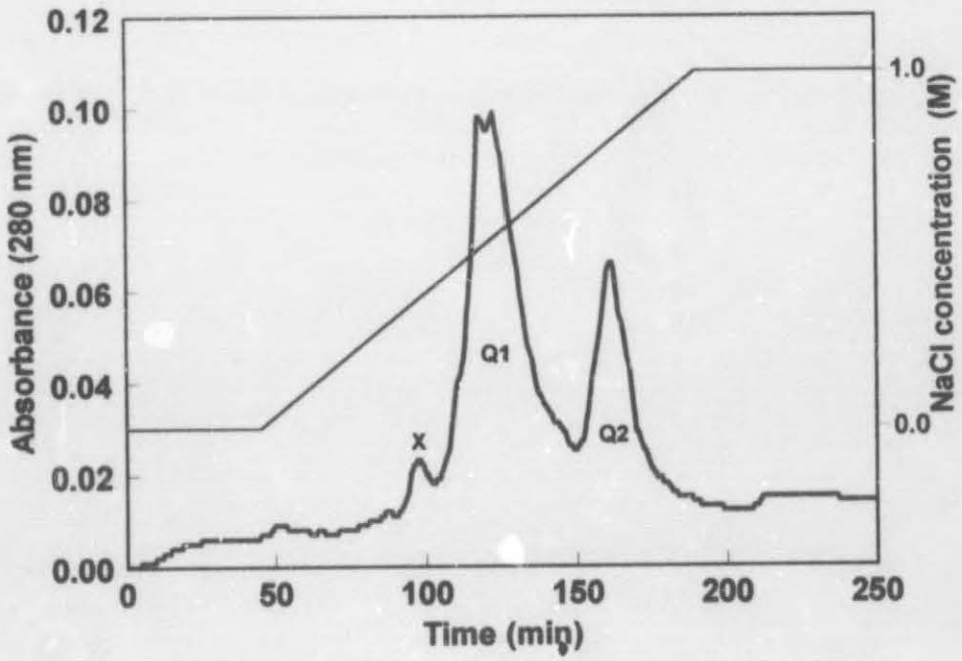

Fig. 4-3 A. Secondary separation of elicitor active peaks of the *P. s.* pv. *syringae* NV bSSN.

Chromatography was carried out as described in Materials and Methods. The salt gradient (indicated in blue) is expressed in terms of the progressive addition of NaCl to the starting buffer (10 mM MES, pH 6.3, supplemented with 0.1 mM PMSF) for the elution of column-bound components. Fractions were collected at five minute intervals. Fractions corresponding to the peaks indicated were pooled for further analysis. The shoulder of Q1 (marked X) was not regarded as a significant peak.

Secondary resolution of P6 of the bSSN was consistently unsuccessful. Two very small peaks were displayed on the chromatogram. Even after concentration of their respective fractions by more than 100 fold, the protein concentrations of these peaks were below the detectable level of the Bradford test, and no HR activity or bands on SDS-PAGE were detected (results not shown).

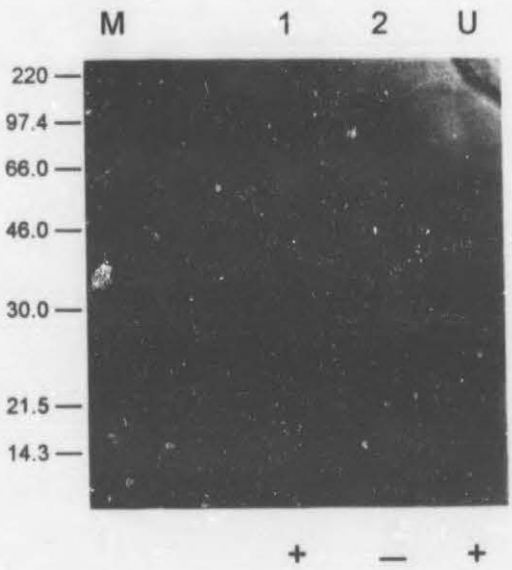

Fig. 4-3 B. Protein profiles on 12 % SDS-PAGE of peaks obtained by secondary ion exchange chromatography of P4&5 of the *P. s.* pv. *syringae* NV bSSN.

Lane M: Rainbow protein standards. **Lanes 1 and 2** correspond with Q1 (11 μg) and Q2 (2 μg) in Fig. 4-3 A. **Lane U:** unresolved bSSN (3.2 μg).

SDS-PAGE and protein determinations were carried out as described in Materials and Methods. The size (kDa) of protein standards are given on the left. The HR phenotype of each protein preparation is indicated at the bottom of each lane.

Resolution of cCSN

Fig. 4-4 A represents the best separation achieved for the cCSN of *P. s.* pv. *syringae* NV on Sepharose CL-6B, using a variety of chromatographic conditions. Six peaks were identified, of which three were badly resolved. Subsequent analyses (HR tests and SDS-PAGE, Fig. 4-4 B) provided the same results as before; the protein profiles of peaks showing elicitor activity were the most complex and could provide no additional evidence on the identity of the sought-after elicitor.

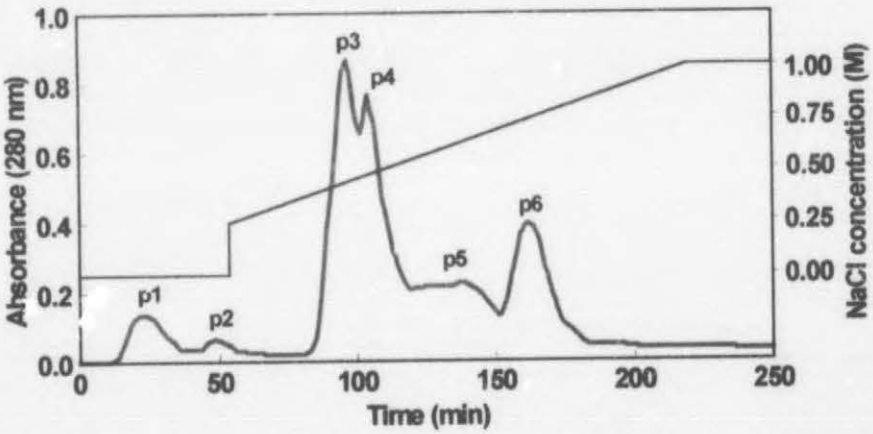

Fig. 4-4 A. Separation by ion exchange chromatography of the cCSN of *P. s.* pv. *syringae* NV grown on NYGB medium.

Chromatography was carried out as described in Materials and Methods. The total protein content of the cCSN loaded on the column was 2.2 mg. The salt gradient (indicated in blue) is expressed in terms of the progressive addition of NaCl to the starting buffer (10 mM MES, pH 6.3, supplemented with 0.1 mM PMSF) for the elution of column-bound components. Fractions were collected at five minute intervals. Fractions corresponding to the peaks indicated were pooled for further analysis.

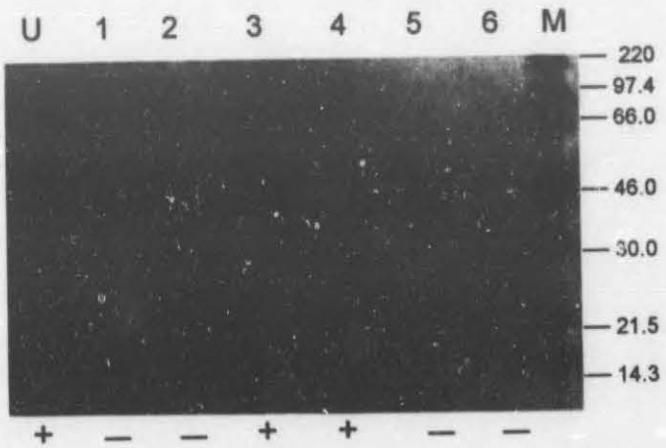

Fig. 4-4 B. Protein profiles on 12 % SDS-PAGE of peaks obtained by ion exchange chromatography of the *P. s.* pv. *syringae* NV cCSN .

Lane U: unresolved bSSN (3.2 µg). **Lanes 1 to 6** correspond with p1 to p6 in Fig. 4-4 A. **Lane M:** Rainbow protein standards.

SDS-PAGE and protein determinations were carried out as described. The total protein content in the aliquot of each of the (20 fold concentrated) peaks loaded on the gel was: p1, 4.6 µg; p2, 1.2 µg; p3, 63 µg; p4, 29 µg; p5, 1.1 µg and p6, 0.1 µg. Sizes (kDa) of protein standards are given on the left. The HR phenotype of each protein preparation is indicated at the bottom of each lane.

HR testing of cCSN from *P. s.* pv. *syringae* NV grown on minimal media indicated the elicitor to be absent or present only in very low concentrations. The simplicity of the SDS-PAGE protein profiles of these preparations, however, rendered them suitable for partial purification of the elicitor protein by ion exchange chromatography. The result of one such a separation is shown in Fig. 4-5. A good resolution of five peaks was obtained. However, subsequent analysis (HR testing and SDS-PAGE) of concentrated peaks yielded no meaningful results. No elicitor activity was detected and only one band of ± 46 kDa could be distinguished on SDS-PAGE. This band was present in peaks 4 and 5 of the separation, as well as in the unresolved cCSN (results not shown).

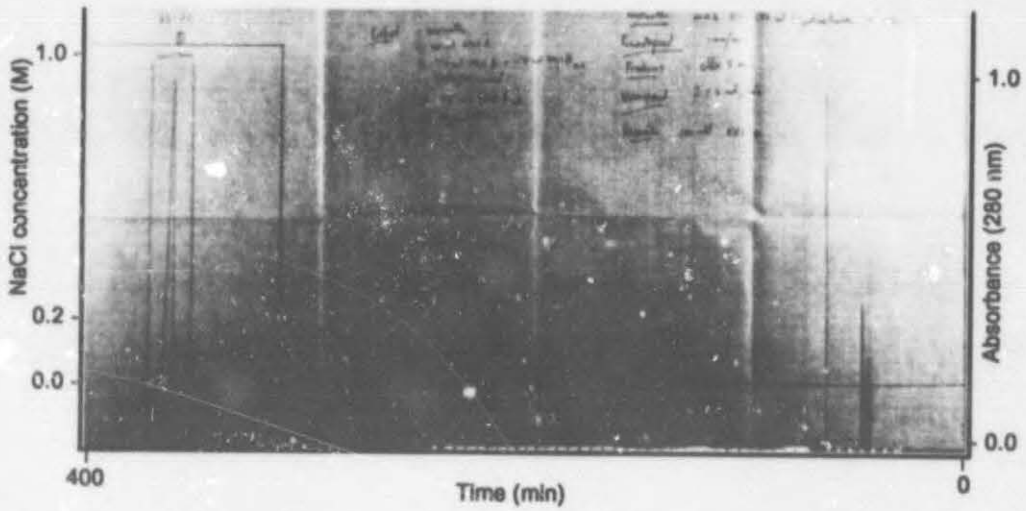

Fig. 4-5. Separation by ion exchange chromatography of the cCSN of *P. s.* pv. *syringae* NV grown on M9 minimal medium, supplemented with D-fructose.

Chromatography was carried out as described in Materials and Methods. A total amount of 0.6 mg protein was loaded on the column. The elution profile consisted of one bed volume of starting buffer (MES A; 10 mM MES, pH 6.3, supplemented with 0.1 mM PMSF). This was followed by a concave salt gradient formed by mixing of three bed volumes each of MES A and MES $B_{0.2}$ (MES A + 0.2 M NaCl). The last column-bound component was eluted by changing to a discontinuous salt gradient through the application of three bed volumes of MES $B_{1.0}$ (MES A + 1 M NaCl). Fractions were collected at five minute intervals. Fractions corresponding to the peaks indicated were pooled for further analysis.

This result (which was repeatedly obtained for all separations of minimal media culture supernatants) could be attributed to degradation of the apparently small quantity of elicitor protein present in the fraction during the concentration process. It nevertheless indicated that culture supernatants of *P. s.* pv. *syringae* NV grown on minimal media did not comprise suitable sources of elicitor protein for purification purposes using the present methodology.

Plasmid isolation from successful *E. coli* DH5α transformants

Results of miniprep plasmid isolations from clone pSYH10-a and control clone pBS-a are given in Table 1-3. Ratios of $A_{260}/A_{280} > 1.8$ indicated contamination with single-stranded DNA or RNA (Maniatis *et al.* 1989). This would, however, not pose any problems for subsequent DNA analyses.

Restriction digestion of the isolated plasmid DNA with *Hind*III was expected to yield one band (2958 bp) in the case of pBluescript and two bands (3414 and 918 bp respectively) in the case of pSYH10, as illustrated in Fig. 4-6.

This result was indeed obtained, as shown by agarose gel electrophoresis of digestion products (Fig. 4-7). The estimated sizes of bands obtained, calculated from the λ-DNA/*Pst*1 digest calibration curve for this gel (not shown), were 3000 bp for pBluescript and 3500 and 900 bp respectively for the two fragments of pSYH10.

Table 4-3. Results of miniprep plasmid isolation.

Clone	A_{260}	A_{280}	A_{260}/A_{280}*	[DNA] (μg/μl)
pSYH10-a	0.1561	0.0639	2.4415	0.0937
pBS-a	0.1094	0.0497	2.2006	0.0656

* Pure DNA preparations have A_{260}/A_{280} of 1.8

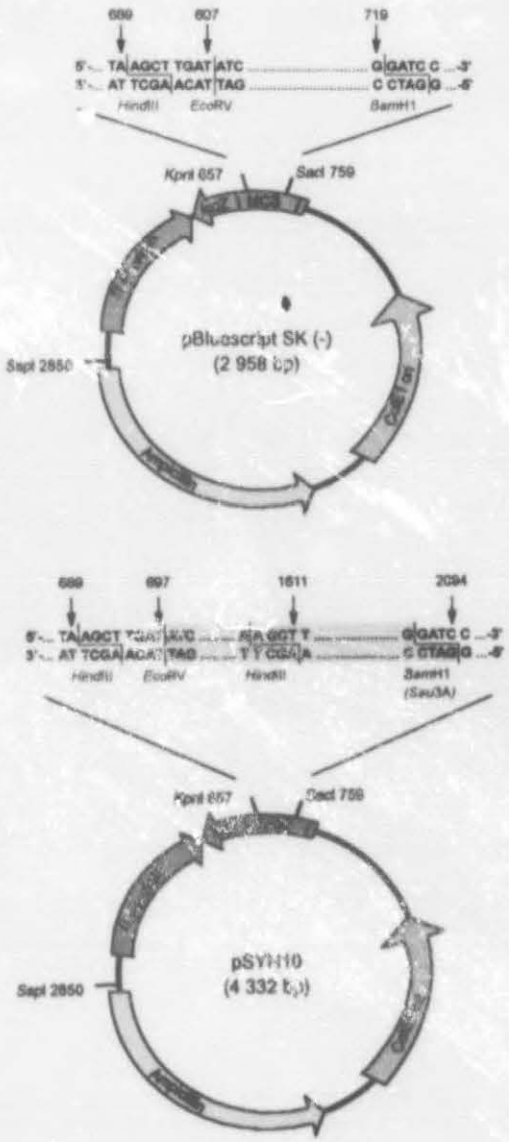

Fig. 4-6. Plasmid maps illustrating some of the features of pBluescript SK(-) and pSYH10 (compiled from Short *et al.* 1988 and He *et al.* 1993).

The unmodified plasmid has only one *Hind*III recognition site, at position 689. Restriction digestion with this endonuclease will subsequently yield a linear DNA fragment of 2 958 bp. A *Sau*3A/*Eco*RV fragment (1 400 bp) of the *P. s.* pv. *syringae* 61 *hrp/hrmA* gene cluster was cloned into pBluescript as indicated (in red) to form pSYH10. Since this insert contains a *Hind*III recognition site, digestion of the recombinant plasmid with this enzyme will yield two fragments of 918 and 3414 bp respectively.

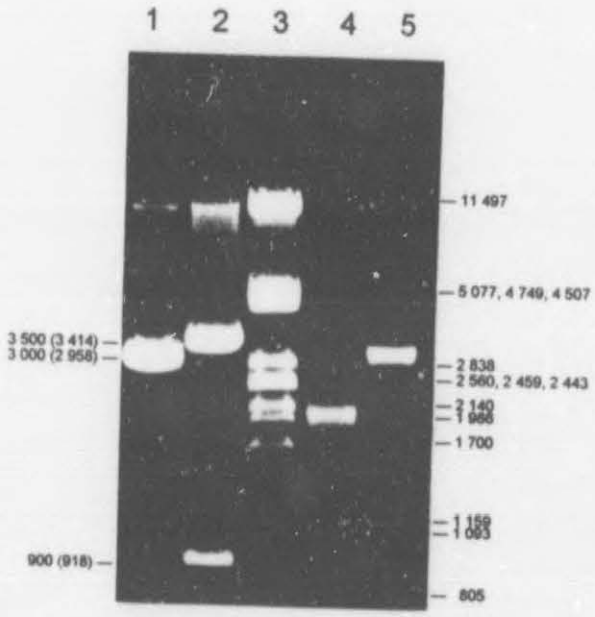

Fig. 4-7. Agarose gel electrophoresis of plasmid DNA isolated from clones pSYH10-a and pBS-a.

Lane 1: pBS-a (*Hind*III digest, 1 µg), **lane 2:** pSYH10-a (*Hind*III digest, 1 µg), **lane 3:** molecular mass marker fragments (*Pst*1 digest of λ-DNA, 1 µg), **lane 4:** pBS-a (uncut, 200 ng; 2958 bp), **lane 5:** pSYH10-a (uncut, 400 ng; 4332 bp).

Plasmid isolation, restriction digests and electrophoresis were carried out as described in Materials and Methods. Sizes (bp) of molecular marker fragments are given on the right. The sizes of bands obtained from plasmid digestion are given on the left. The estimated molecular mass obtained from the M, calibration curve for this gel is given first, with the theoretical value in parenthesis. For both of the uncut plasmids, the three typical topoisomeric forms (in order of increasing mobility); the linear duplex, nicked circle and closed circle (Ausubel *et al.* 1995), can be distinguished.

Transformation experiments were thus successful and clone pSYH10-a could be used for the expression of recombinant harpin$_{Pss}$. Clone pBS-a constituted the necessary control for protein expression experiments, as it would yield profiles identical to that of pSYH10-a, with the exception of proteins expressed as a result of the introduction of the *P. s.* pv. *syringae* 61 *hrpZ2* containing fragment into the phagemid vector.

Protein profiles and elicitor activity of *E. coli* fractions

Protein profiles obtained by SDS-PAGE of the four fractions from clone pSYH10-a and control clone pBS-a are given in Fig. 4-8. Lanes containing SSN, SP and bP fractions were somewhat overloaded and thus not very informative. A clear difference could, however, be noticed between the bSSN fractions of pSYH10-a and the control. The former bacterium produced an "extra" protein of between 30 and 46 kDa in size (according to protein standards).

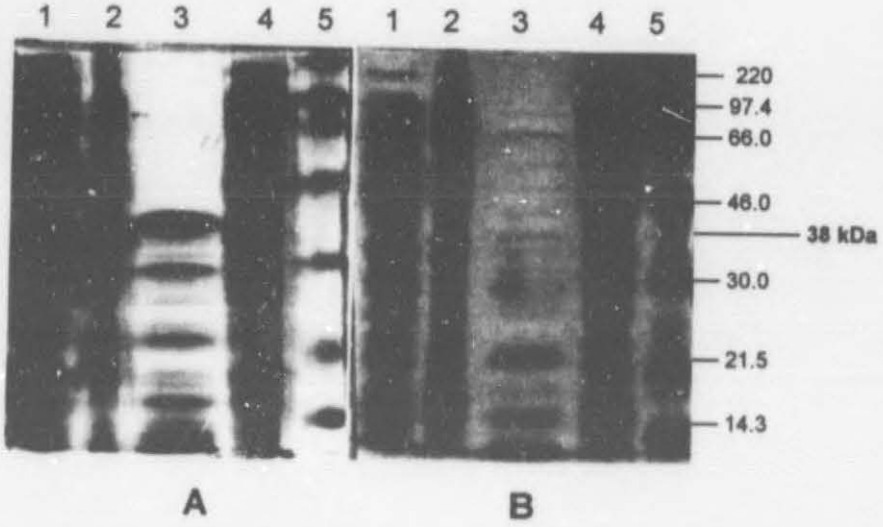

Fig. 4-8. Protein profiles of bacterial fractions from recombinant *E. coli* DH5α clones **A.** pSYH10-a and **B.** pBS-a, as obtained by 12 % SDS-PAGE.

Lane 1: SSN, **lane 2:** SP, **lane 3:** bSSN, **lane 4:** bP, **lane 5:** Rainbow protein standards.

SDS-PAGE was carried out as described in Materials and Methods. The total amount of protein loaded in each lane, determined by the Bradford method, was: pSYH10-a SSN, 230 mg; pSYH10-a bSSN, 14 mg; pBS-a SSN, 23 mg and pBS-a bSSN, 6 mg. Protein concentrations for pellet fractions could not be determined accurately, as resuspended pellets did not form homogenous suspensions. The sizes (kDa) of protein standards are given on the right.

Using the calibration curve for this particular gel (Fig. 4-9), the molecular weight of this band was calculated to be 38 kDa. In the original study, the size of harpin$_{Psa}$ was reported to be 34.7 kDa, as determined by sequence analysis and confirmed by mass spectrometry. However, the size of the protein, estimated by 12 % SDS-PAGE, was 42 kDa. This led the authors to believe that the protein migrates abnormally in SDS gels (He *et al.* 1993). The intensity of our 38 kDa band, relative to others in the same fraction, was typical of over-expressed, recombinant proteins. It was therefore concluded that this band constituted the recombinant harpin$_{Pss}$, despite the fact that its apparent molecular weight differed from the original report. This difference could possibly be attributed to the use of different protein standards in the two studies.

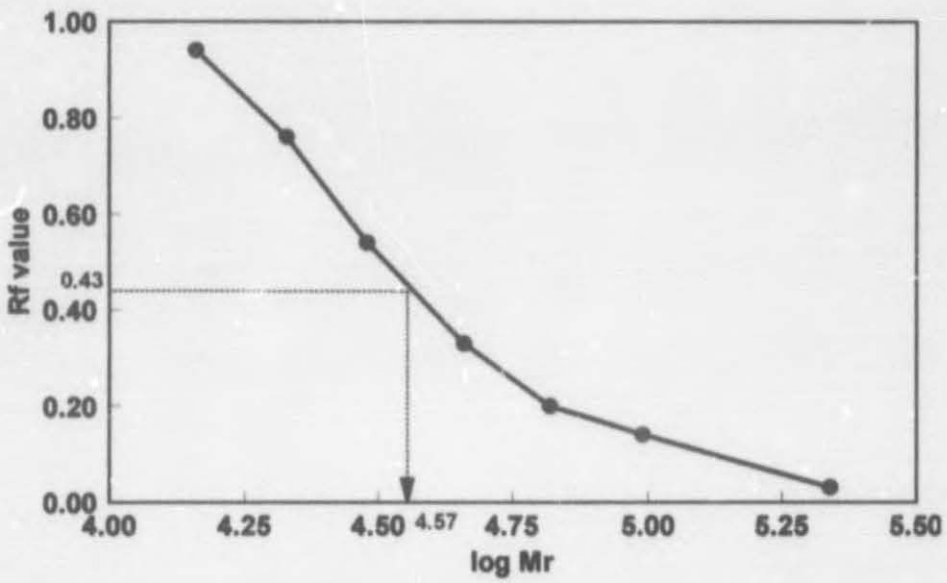

Fig. 4-9. Rainbow marker calibration curve for the determination of molecular weight of proteins separated by 12 % SDS-PAGE

SDS-PAGE was carried out as described in Materials and Methods. The R$_f$ value of a specific band is expressed as the mobility of the band (mm) divided by the mobility of front (mm). The non-linear relationship between the electrophoretic mobility and log M, obtained for Rainbow markers is the result of differential colour-derivitazation of the respective proteins. Since the form of this curve is not described by any regular function, the log M, of the putative recombinant harpin$_{Pss}$ was read from the curve using a grid with a resolution of 0.01 units on both the x- and the y- axes.

Results of HR tests (shown in Fig. 4-10) confirmed the identity of the 38 kDa band. Elicitor activity was exhibited only by fractions of clone pSYH10-a containing this band (SSN and bSSN). This agreed with previously published characteristics of $harpin_{Pss}$, namely its extracellular location and heat-stability (He *et al.* 1993). No elicitor activity was recorded for any of the fractions of the control clone. The partially purified bSSN of pSYH10-a was subsequently selected as starting material from which $harpin_{Pss}$ was to be purified to homogeneity for antibody production.

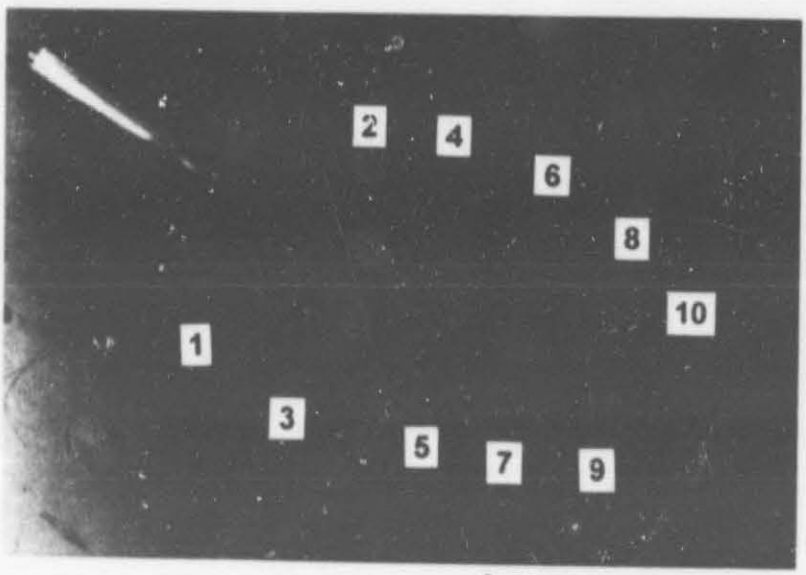

Fig. 4-10. Tobacco leaf showing the HR phenotypes (elicitor acitivity) of recombinant *E. coli* DH5α fractions.

Panels 1-4 were respectively infiltrated with the SSN, SP, bSSN and bP fractions of the control clone pBS-a and **panels 5-8** with the corresponding fractions of clone pSYH10-a. **Panel 9** was infiltrated with 10 mM MES (pH 5.5), supplemented with 0.1 mM PMSF. **Panel 10** was infiltrated with a suspension (~ 10^8 cells/ml) of *E. coli* DH5α(pSYH10) in sterile distilled water.

HR tests were conducted as described in Materials and Methods. The photograph was taken 48 hours after infiltration. Duplicate infiltrations, done in a similar fashion on a different plant, yielded identical results. The absence of symptoms in panel 10 confirms that *E. coli* does not have the ability to export the recombinant $harpin_{Pss}$ after production.

Purification of recombinant harpin$_{Pss}$

Purification by non-denaturing PAGE

Fig. 4-11 shows the result of 12 % SDS-PAGE of the harpin$_{Pss}$ preparation obtained by ND-PAGE and electro-elution. Different quantities of protein were loaded on the gel to assess whether the sample was indeed homogeneous. A band corresponding in size to the 38 kDa band in the pSYH10-a bSSN was detected in all lanes. However, in lanes containing larger quantities of protein, a second smaller band of lower intensity could also be seen. According to the molecular weight calibration curve for this gel (not shown), the size of the smaller band was calculated to be 14 kDa. The harpin$_{Pss}$ preparation obtained by this method was therefore not homogeneous.

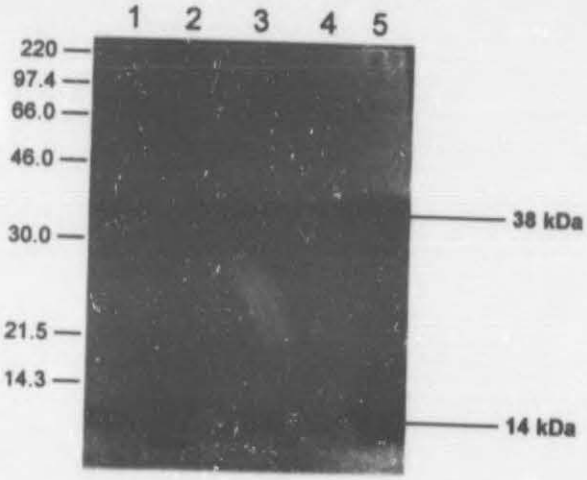

Fig. 4-11. SDS-PAGE of harpin$_{Pss}$ purified by non-denaturing PAGE and electro-elution.

Lane 1: Harpin$_{Pss}$ (4.5 µg), **lane 2:** Rainbow molecular weight markers, **lane 3:** Harpin$_{Pss}$ (1.5 µg), **lane 4:** Harpin$_{Pss}$ (0.38 µg), **lane 5:** pSYH10-a bSSN (29 µg).

The purification protocol was carried out as described in Materials and Methods. The total amount of protein loaded in each lane, determined by the Bradford method, is given in parenthesis. The sizes (kDa) of protein standards are given on the left.

Another apparent disadvantage of this method was the low yield of specific protein. The total quantity of eluted protein amounted to 0.47 mg, or 3.7 % of the total protein content in the bSSN loaded on the ND-PAGE gel. Although the expression efficiency of the recombinant protein in this system could not be quantified, a visual assessment of SDS-PAGE protein profiles suggested the percentage of recombinant protein in the partially heat-purified bSSN to be higher than 4 %.

Purification by ProSieve agarose gel electrophoresis

SDS-PAGE (12 %) of ProSieve agarose gel purified harpin$_{Pss}$ (Fig. 4-12) showed a single band in both lanes where different quantities of the preparation were loaded. It corresponded in size to the 38 kDa band in the pSYH10-a bSSN. The harpin$_{Pss}$ preparation obtained by this method therefore appeared to be homogeneous, rendering this purification protocol more efficient than the former.

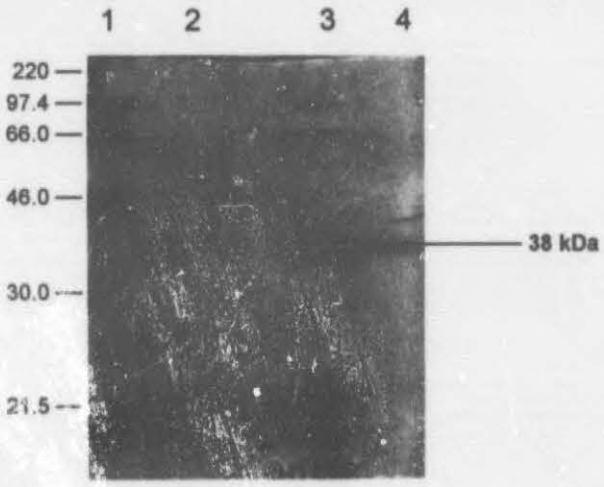

Fig. 4-12. SDS-PAGE of harpin$_{Pss}$ purified from a ProSieve agarose gel.

Lane 1: Rainbow molecular weight markers, **lane 2:** Harpin$_{Pss}$ (1.7 µg), **lane 3:** pSYH10-a bSSN (36 µg), **lane 4:** Harpin$_{Pss}$ (0.85 µg).

The purification protocol was carried out as described in Materials and Methods. The total amount of protein loaded in each lane, determined by the Bradford method, is given in parenthesis. The sizes (kDa) of protein standards are given on the left.

The yield of harpin$_{Pss}$ by this method amounted to 7.1 mg, or 58 % of the total protein content of the bSSN loaded on the ProSieve gel. This figure seemed to be too high, but was definitely closer to the true percentage of recombinant protein in the bSSN than obtained with the ND-PAGE method.

Western blot analyses using rabbit anti-harpin$_{Pss}$ antisera

Evaluation of antiserum specificity and sensitivity

Results of an evaluation of the specificity and sensitivity of the day 18 anti-harpin$_{Pss}$ antiserum is given in Fig. 4-13 A. A band, corresponding in size to the 38 kDa band in the *E. coli* DH5α (pSYH10) bSSN, was recognized at all but the 1/5000 antiserum dilutions used (this dilution was not included in the figure).

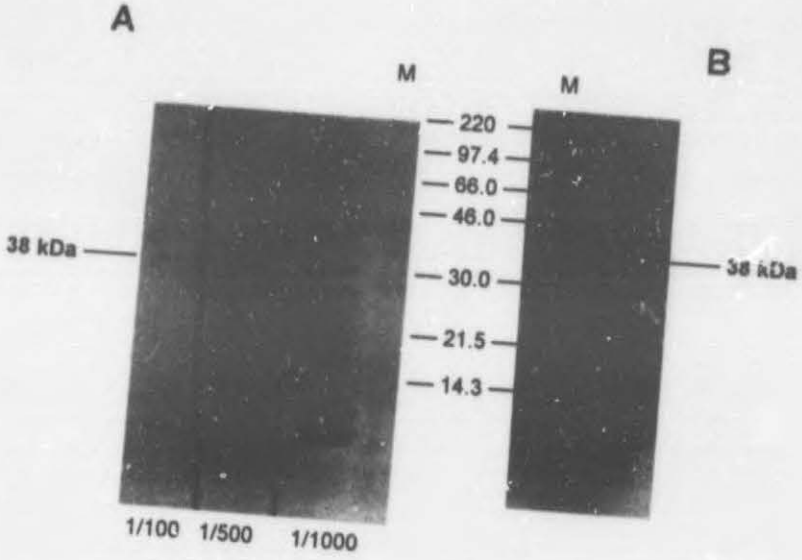

Fig. 4-13. Evaluation of the specificity and sensitivity of day 18 rabbit anti-harpin$_{Pss}$ antiserum by **A.** Western blot analysis of *E. coli* DH5α (pSYH10) bSSN and **B.** corresponding Coomassie staining of the SDS-PAGE result.

SDS-PAGE and immunoblotting were performed as described in Materials and Methods. A constant amount (total protein content, 4 µg) of *E. coli* DH5α (pSYH10) bSSN was loaded in each lane, except for those containing Rainbow molecular mass marker proteins (M). The dilution of the primary antibody (anti-harpin$_{Pss}$ antiserum) used in each instance is indicated at the bottom of the respective nitrocellulose strips. The sizes (kDa) of protein standards are given on the right.

Comparison with the section of the SDS-PAGE gel that was stained (Fig. 4-13 B), as well as calculation of the estimated molecular mass of this recognized band from the calibration curve for the gel (not shown), confirmed it to be the recombinant harpin$_{Pss}$. The antigen used for antibody production was thus recognized by the day 18 antiserum. The faintness of the recognized band at a 1/1000 dilution, and the presence of a number bands resulting from non-specific antibody interactions at a 1/100 dilution, were typical for a polyclonal antiserum derived early in the immune response.

Repetitions of this experiment with antisera collected on days 32 and 46 did, however, not demonstrate the desired improvement in specificity and sensitivity to the antigen. Harpin$_{Pss}$ was still recognized by the day 32 antiserum in the bSSN of *E. coli* DH5α (pSYH10) bSSN (Fig. 4-14 A). However, a huge increase in the number of bands resulting from non-specific recognition was noted for this serum. This marked a decrease in the specificity of the antiserum. The sensitivity of the antiserum for its antigen did also not improve, as the intensity of the harpin band recognized by 1/500 and 1/1000 dilutions of the day 32 serum did not increase significantly compared to the result obtained with the same dilutions of day 18 serum (compare Figs. 4-13 A and 4-14 A).

The situation was even worse with the day 46 antiserum. At serum dilutions of 1/2000 and 1/1000, the harpin$_{Pss}$ band in the bSSN of *E. coli* DH5α (pSYH10) was not recognized at all (Fig. 4-14 B). Concurrently, the intensity of larger (± 66-90 kDa) and smaller (± 14-23 kDa) bands, previously attributed to "non-specific antibody interactions" with the components of this sample, increased even more than was the case in the recognition of these bands by day 18 and 32 sera (compare Figs. 4-13, 4-14 A and 4-14 B).

The most probable cause for this deterioration in the immune response to harpin$_{Pss}$ was suggested from the banding pattern in lane 2 on the blots in Fig. 4-14 A. The lyophilized, ProSieve gel purified recombinant harpin$_{Pss}$ used for preparation of the antigen suspension was electrophoresed here. From the multiple, low molecular mass bands recognized with the day 32 antiserum, it was evident that serious degradation of the protein occurred during storage. Electrophoresis was performed

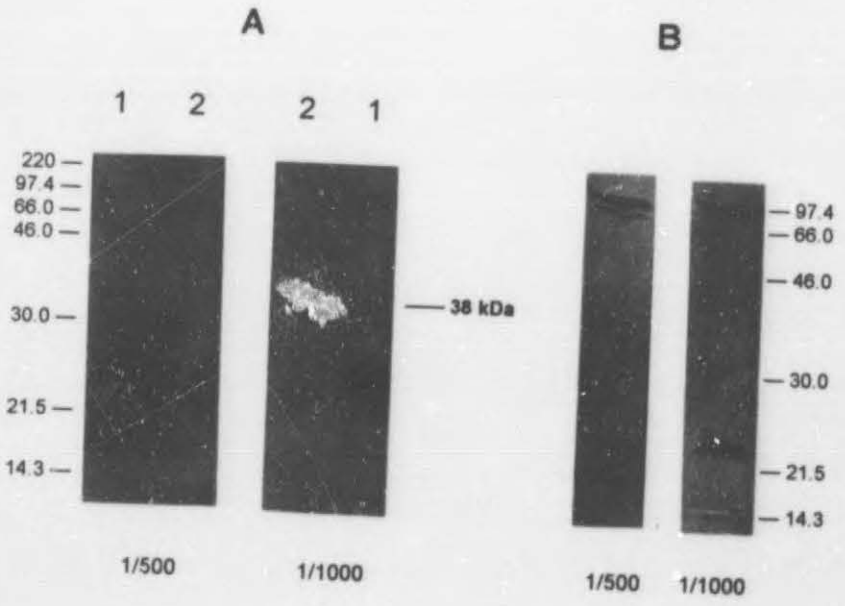

Fig. 4-14. Evaluation of the specificity and sensitivity of **A.** day 32 and **B.** day 46 rabbit anti-harpin$_{Pss}$ antisera.

A. Lane 1: *E. coli* DH5α (pSYH10) bSSN (29 µg) and **lane 2:** Lyophilized recombinant harpin$_{Pss}$, purified from ProSieve gel (27 µg).

B. *E. coli* DH5α (pSYH10) bSSN (24 µg) was loaded in each of the two lanes.

SDS-PAGE and immunoblotting were performed as described in Materials and Methods. The serum dilution used is indicated at the bottom of the respective membrane strips. Rainbow protein standards were included in all gels. Unfortunately, the coloured bands faded to invisibility. The sizes (kDa) of protein standards are therefore noted accurately in positions corresponding to mobility of each standard in the particular gel. Coomassie staining of SDS-PAGE results are not shown.

less than a week after preparation of the harpin$_{Pss}$-NB complex to be used in the second round of immunization. Both the second and third rounds of immunization were thus performed with degraded antigen instead of the original, intact protein.

Harpin$_{Pss}$ degradation would result in (a) adsorption of shorter polypeptides to the surface of NB and/or (b) the exposure of more antigenic regions on the surface of the immune carrier than would be the case in the presence of intact antigen. Both of

these scenarios would result in preferred amplification of "non-specific" antibody clones in the polyclonal antisera produced during the development of the immune response. Non-specific antibodies against harpin$_{Pss}$ degradation products would consequently recognize lower molecular mass bands. Non-specific antibody clones against NB antigenic determinants could react with a number of bacterial proteins, as *E. coli* and *Salmonella minnesota* are both Gram-negative bacteria, sharing a number of common cell wall components (Bellstedt 1988). The increased specificity of day 32 and 46 antisera for protein components larger and smaller than harpin$_{Pss}$ can thus be explained satisfactorily against this background.

From the abovementioned results it was concluded that the best specificity-sensitivity combination was displayed by the day 18 anti-harpin$_{Pss}$ antiserum. This serum was, however, not suitable for use in a purification protocol for the *P. s.* pv. *syringae* NV elicitor based on specific antigen-antibody binding. It could, nevertheless, be used in attempts to pinpoint the position of the *P. s.* pv. *syringae* NV elicitor in the protein profiles of elicitor active bacterial fractions, from which important information (e.g. the size of this protein) could possibly be gained.

Western blot analysis of P. s. *pv.* syringae *NV fractions*

The result of Western blot analysis of *P. s.* pv. *syringae* NV fractions with the day 18 anti-harpin$_{Pss}$ antiserum is shown in Fig. 4-15 A. Numerous "non-specific" bands were obtained, especially in fractions with a high total protein content and complex protein profiles. A duplicate analysis on aliquots of the bSSN of *E. coli* DH5α (pSYH10) and *P. s.* pv. *syringae* NV, using the control (day 0) antiserum showed that the occurrence of non-specific bands was limited to high (> 70 kDa) and low (< 25 kDa) molecular mass regions (Fig. 4-15 B). The intermediate mass range (30 45 kDa), in which harpin$_{Pss}$ was situated, and where the elusive *P. s.* pv. *syringae* NV was believed to be found, seemed to be free of non-specific band recognition.

This enabled the identification of a band, slightly larger than the recombinant harpin$_{Pss}$ in the *E. coli* DH5α (pSYH10) bSSN, in all *P. s.* pv. *syringae* NV fractions. Its intensity in a specific fraction was dependent on the protein content of the fraction aliquot loaded on the gel, but also increased when fractions were concentrated by

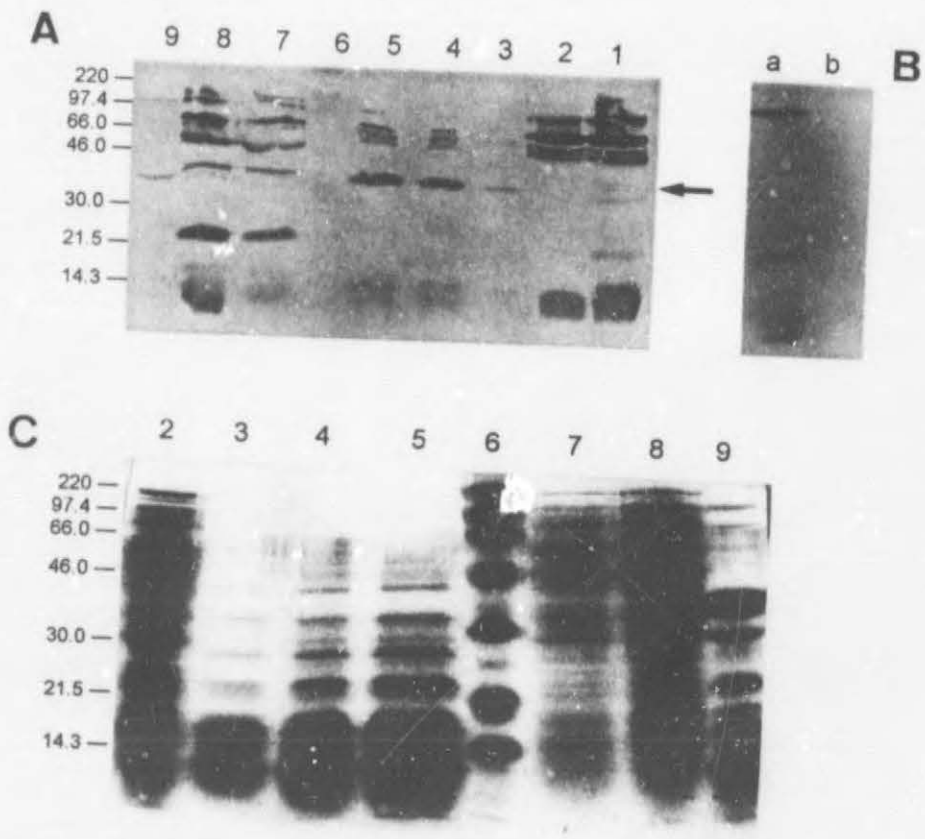

Fig. 4-15. Western blot analysis of **A.** *P. s.* pv. *syringae* NV fractions, using day 18 anti-harpin$_{Pss}$ antiserum and **B.** the bSSN of *E. coli* DH5α (pSYH10) and cCSN of *P. s* pv. *syringae* NV, using day 0 anti-harpin$_{Pss}$ antiserum. Coomassie staining of the corresponding SDS-PAGE result of **A.** is given in **C.**

A. Lane 1: Total cell sonicate (ND), **lane 2:** SSN (32 µg), **lane 3:** bSSN (3.8 µg), **lane 4:** bSSN (concentrated by rotor evaporation, 12 µg), **lane 5:** bSSN (concentrated by lyophilization, 19 µg), **lane 6:** Rainbow protein standards, **lane 7:** cCSN (10 fold concentrated, 4.4 µg), **lane 8:** cCSN (30 fold concentrated, 16 µg), **lane 9:** *E. coli* DH5α (pSYH10) bSSN (17 µg).

B. Lane a: *P. s.* pv. *syringae* NV cCSN (17 µg) and **lane b:** *E. coli* DH5α (pSYH10) bSSN (18 µg).

C. Lane numbers and protein concentrations are the same as in **A.**

SDS-PAGE and immunoblotting were performed as described. The respective antisera were both used at a dilution of 1/500. The gels used for immunoblotting and Coomassie staining were identical, with the exception that the total *P. s.* pv. *syringae* NV cell sonicate was omitted in the latter. The total amount of protein loaded in each lane is given in parenthesis. This value could not be determined for the total cell sonicate, due to the turbidity of the sample. The sizes (kDa) of protein standards are given on the left. The putative NV elicitor band is indicated by the arrow on the right.

heat-treatment (boiling of SSN to obtain bSSN, five fold concentration of bSSN by rotary evaporation). All of the *P. s.* pv. *syringae* NV fractions exhibited elicitor activity in tobacco plants (result not shown). From these results it was concluded that the "harpin$_{Pss}$-like" band produced by *P. s.* pv. *syringae* NV constitutes the sought-after harpin elicitor of this pathogen. The estimated size of this putative NV elicitor, was calculated to be 39 kDa.

Despite the fact that a putative NV harpin had been identified, protein profiles visualized by Coomassie staining of the SDS-PAGE result (Fig. 4-15 C), were too complex and the quantity of the particular band too small to allow purification by the excision of the relevant gel section and electro-elution. Finer resolution of elicitor active *P. s.* pv. *syringae* NV fractions was consequently attempted by ion exchange chromatography.

Western blot analysis of elicitor active P. s. *pv.* syringae *NV fractions resolved by ion exchange chromatography*

The result of Western blot analysis of subfractions of the *P. s.* pv. *syringae* NV bSSN and cCSN, obtained by ion exchange chromatography, is given in Fig. 4-16 A.

The two peaks (Q1 and Q2) obtained after the secondary separation of the elicitor active P4&5 of the bSSN, as well as all of the peaks (p1-p6) obtained from the resolution of the cCSN were analyzed. A 1/500 dilution of the day 18 anti-harpin$_{Pss}$ antiserum were used in both cases. Coomassie staining of the SDS-PAGE protein profiles of these samples were given earlier (Fig. 4-3 B and 4-4 B).

A band, corresponding in size to harpin$_{Pss}$ and to the putative NV elicitor was detected in all elicitor active subfractions of the *P. s.* pv. *syringae* NV bSSN and cCSN. This band did not appear in any of the subfractions displaying a negative HR result. It also did not appear in any lane of the "control" blot (Fig. 4-16 B), which was obtained through the use of day 18 (1/500 diluted) anti-lysozyme antibodies. It was therefore concluded that this band represented the same putative *P. s.* pv. *syringae* NV elicitor identified previously (see Fig. 4-15). Photographs of blots were, unfortunately, taken a few months after these experiments were conducted. This led to a reduction in the intensity of reaction products, rendering published photographs

an underestimation of the true result. Time constraints prevented the repetition of this experiment in order to obtain a clearer publishable result.

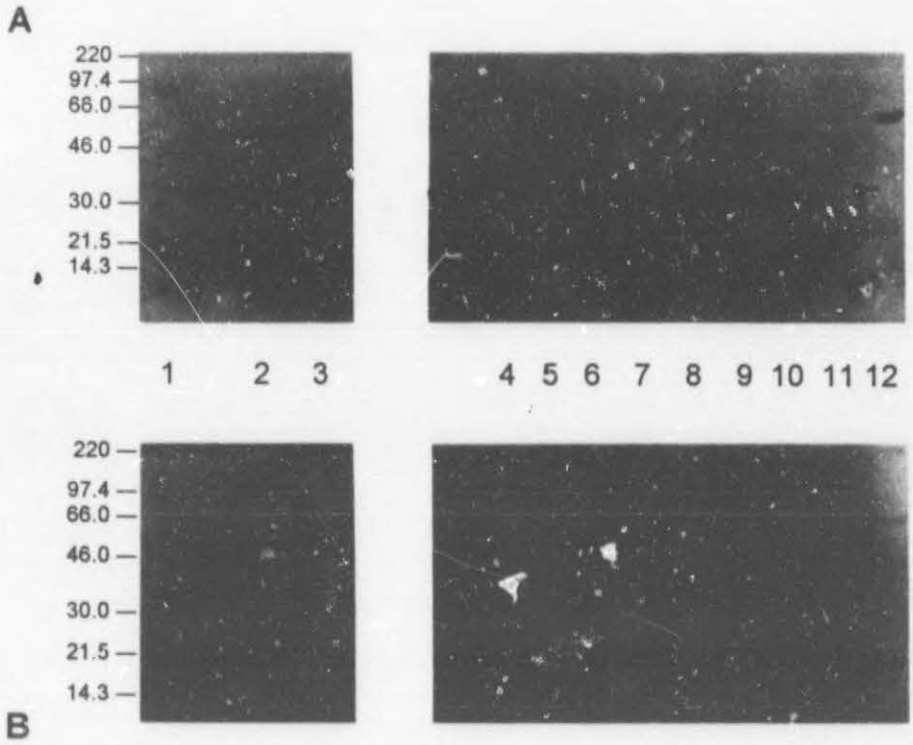

Fig. 4-16. Western blot analysis of elicitor active *P. s.* pv *syringae* NV fractions resolved by ion exchange chromatography, using **A.** day 18 anti-harpin$_{Pss}$ antiserum and **B.** day 18 anti-lysozyme antiserum as control.

Lanes 1 and 5: Rainbow protein standards, **lanes 2 and 3:** *P. s.* pv. *syringae* NV bSSN secondary peaks Q1 and Q2, **lane 4:** *E. coli* DH5α (pSYH10) bSSN, **lanes 6-11:** peaks p1-p6 of *P. s.* pv. *syringae* NV cCSN, **lane 12:** unresolved *P. s.* pv. *syringae* NV cCSN.

SDS-PAGE and immunoblotting were performed as described in Materials and Methods. Coomassie stained protein profiles of these samples were given in Fig. 4-3 B and 4-4 B. The amounts of protein loaded in each lane of the gel were the same as indicated there. The sizes (kDa) of protein standards are given on the left. The position of the harpin$_{Pss}$ band is indicated by the arrow and the position of the putative *P. s.* pv. *syringae* NV elicitor band by an asterisk in elicitor active samples.

DISCUSSION

The cultivation of *P. s.* pv. *syringae* NV on different media led to the first unexpected results in this study. Elicitor activity was detected readily in fractions of the bacterium cultivated on NYGB, despite previous reports that the expression of *hrp* genes were repressed (but not totally abolished) in peptone-containing media (Lindgren *et al.* 1989, Yucel *et al.* 1989, Rahme *et al.* 1992 and Xiao *et al.* 1992). Concomitantly, no HR activity could be detected in this study in corresponding fractions of the bacterium grown in minimal media used by these and other authors (Huynh *et al.* 1989, He *et al.* 1993) for the induction of genes necessary for HR elicitation. Taking into account the weak growth of the bacterium on these media (compared to growth on NYGB), a possible explanation for this result is that the elicitor was being produced, but in too low quantities to be detected (since the HR is a concentration dependent phenomenon). A better result would probably have been obtained had the cultures been left to grow for a longer time or alternatively, been grown for an initial period on NYGB to a reasonable density, harvested and transferred to a minimal medium for elicitor induction, as described by Grimm *et al.* (1989). Despite these discrepancies, the results of initial experiments on *P. s.* pv. *syringae* NV, grown on NYGB, still provided enough evidence on similarities between a possible *P. s.* pv. *syringae* NV elicitor and harpin$_{Pss}$ to continue with the approach that was chosen for this study.

Failure to detect elicitor activity in fractions of *P. s.* pv. *syringae* NV grown on minimal media had a significant effect on efforts to obtain partially purified elicitor preparations through the use of ion exchange chromatography. Subfractions of NYGB culture supernatants were resolved successfully to a certain extent, as elicitor activity was repeatedly confined to one or two fractions. However, the complexity of the culture medium appeared to be the factor limiting further progress in this regard. Had proper growth and elicitor production be achieved on minimal media, the absence of protein or peptide components in these media might even have allowed the purification of the elicitor protein from cCSN without the use of antibodies.

The real setback in this study was, however, the poor quality of the anti-harpin$_{Pss}$ antiserum. Problems in this regard were rooted in the purification of the recombinant

harpin$_{Pss}$. The protocol used by He *et al.* (1993) had to be modified, as the gel system used by them (NuSieve agarose, FMC) was not available for this study. After unsuccessful attempts to purify the protein by the conventional electro-elution procedure, the opportunity to acquire a derivitized gel system (ProSieve agarose, FMC) did arise. The availability of this gel was, however, discontinued by the producer shortly thereafter, and the purification of the recombinant harpin$_{Pss}$ could not be repeated satisfactorily.

The available quantity of purified antigen was consequently the determining factor in the choice of methodology followed for the production of the antiserum. Despite the fact that naked bacteria have been used successfully as immune carriers for the production of highly specific antibodies against protein antigens (Bellstedt *et al.* 1987, 1993), this system had not been evaluated thoroughly for use against proteins of bacterial origin. The more conventional method of using Freund's adjuvant (Freund and Bonanto 1944) could possibly have resulted in antisera with lower levels of non-specific antibody clones, induced by immune carrier antigenic determinants. However, this alternative method requires much larger quantities of antigen for immunization. With the limited quantity of purified harpin$_{Pss}$ available, it was debatable whether a specific anti-harpin$_{Pss}$ antiserum of the desired sensitivity could be raised at all with this method.

The fact that a protein band similar in size to harpin$_{Psc}$ could be identified consistently in elicitor active fractions and subfractions of *P. s.* pv. *syringae* NV despite the limited capabilities of the anti-harpin$_{Pss}$ antiserum, was a positive and important result for our continued research efforts. It confirmed that extensive homology on the protein level exists between harpin$_{Pss}$ and the putative harpin elicitor of *P. s.* pv. *syringae* NV. This finding, supported by the many reports on the DNA homology between the *hrp* loci of different *P. syringae* pathovars (Panopoulos *et al.* 1985, Lindgren *et al.* 1986, 1988, Huang *et al.* 1991, Hutcheson *et al.* 1994), provided us with the confidence that the *P. s.* pv. *syringae* NV harpin encoding gene could be located without having to clone the entire *hrp* gene cluster of this strain. The availability of the *P. s.* pv. *syringae* NV harpin gene would permit its over-expression in a recombinant system, by which the isolation and purification of the protein product would be facilitated greatly. Although the classical biochemical

purification strategy presented here has merit, the molecular biology approach holds many advantages; especially in difficult cases such as this one, where the target protein is labile and difficult to assay as a result of the meagre knowledge about its structure and function.

CHAPTER 5

AMPLIFICATION AND CHARACTERIZATION OF A *HRPZ2*-LIKE DNA FRAGMENT FROM THE GENOME OF *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE* ISOLATED FROM A NECTARINE TREE

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ABSTRACT

We have attempted to locate the gene encoding the harpin elicitor of a South African strain (NV) of *Pseudomonas syringae* pv. *syringae*, isolated from a nectarine tree. For this purpose primers, based on the terminal sequences of the harpin$_{Pss}$ encoding (*hrpZ2*) gene of *P. s.* pv. *syringae* strain 61, were used in a polymerase chain reaction with the total genomic DNA of the nectarine pathogen as template. A PCR product, similar in size to harpin$_{Pss}$ was obtained. Partial sequencing of this PCR product, as well as hybridization with the *Bst*X1 fragment of *hrpZ2* under stringent conditions, provided evidence that the *hrpZ2*-like fragment amplified from the *P. s.* pv. *syringae* NV genome constitutes the gene encoding the harpin elicitor of this pathogen.

INTRODUCTION

Studies on the genetic basis of interactions between plants and phytopathogenic bacteria have been conducted for the past thirty years. Ten years ago, Panopoulos and Peet (1985) stated that "... perhaps as many as 100 genes are required ... for bacterial pathogenicity". A set of genes of particular importance in this regard are the *hrp* (*h*ypersensitive *r*eaction/*p*athogenicity) genes. These genes were shown to control the ability of a number of phytopathogenic bacteria to elicit a hypersensitive reaction (HR) in non-host plants and cause pathogenesis in host plants. *Hrp* genes were demonstrated to be clustered, and some to be widely conserved in Gram-negative phytopathogenic bacteria that cause eventual necrosis of their hosts.

These include *Pseudomonas syringae* (Anderson and Mills 1985, Niepold *et al.* 1985, Cuppels 1986, Lindgren *et al.* 1985, 1986, 1988, Somlyai *et al.* 1986, Malik *et al.* 1987, Deasy and Matthyse 1988, Huang *et al.* 1988, Huynh *et al.* 1989), *Pseudomonas solanacearum* (Boucher *et al.* 1987, Huang *et al.* 1990, Gough *et al.* 1992), *Xanthomonas campestris* (Daniels *et al.* 1988, Stall and Minsavage 1990, Arlat *et al.* 1991, Bonas *et al.* 1991, Fenselau *et al.* 1992, Schulte and Bonas 1992) and *Erwinia amylovora* (Bauer and Beer 1987, Steinberger and Beer 1988, Laby and Beer 1992). The *hrp* encoded proteins which are directly responsible for the elicitation of the HR ("harpins") of three phytopathogens (harpin$_{Ea}$ of *Erwinia amylovora* Ea321, Wei *et al.* 1992a; harpin$_{Pss}$ of *Pseudomonas syringae* pv. *syringae* 61, He *et al.* 1993 and harpin$_{Psp}$ of *P. s.* pv. *phaseolicola*, Panopoulos 1995) have been identified, purified and characterized.

Our interest in harpin production by *P. s.* pv. *syringae* stems from the severity of bacterial canker of stone fruit trees, caused by this bacterium, in South Africa. Chemical control of the disease has failed completely and effective strategies for the control of this disease will have to include the selection and breeding of resistance in host trees (Hattingh *et al.* 1989). The identification and purification of a harpin elicitor produced by *P. s.* pv. *syringae* NV, a strain that was isolated locally from a nectarine tree, was undertaken as a first approach towards assisting in such breeding programmes. The objective was to evaluate the use of the purified protein in the development of a screening programme for resistance against *P. s.* pv. *syringae* in commercially important stone fruit cultivars.

Our initial analyses of cellular fractions and culture supernatants of *P. s.* pv. *syringae* NV, revealed that the pathogen produces an extracellular, heat-stable and protease sensitive elicitor of the HR in tobacco (Appel *et al.* 1995, see also chapter 4 of this thesis). These characteristics correspond to those of the *P. s.* pv. *syringae* 61 harpin (He *et al.* 1993). A band similar in size to the *P. s.* pv. *syringae* 61 harpin was also detected with an antibody against this protein in all *P. s.* pv. *syringae* NV elicitor active preparations. However, the NV elicitor could not be purified successfully from such preparations using (this antibody in) classical biochemical methodology. Even if this would have succeeded, the endogenous levels of elicitor produced by the

native bacterium appeared to be so low that routine use of the purified protein would require its over-expression in a recombinant system.

We subsequently decided to direct our efforts towards the location of the gene encoding the harpin of the nectarine pathogen. Our choice of methodology was once again influenced by the availability of the recombinant plasmid, pSYH10, which contains the whole open reading frame (ORF) of *hrpZ2*, the gene encoding $harpin_{Pss}$ (He *et al.* 1993). Instead of following the method used by He *et al.* (1993), which would have involved identification, cloning and subcloning of the entire *P. s. pv. syringae* NV *hrp* gene cluster, it was hoped that the gene (or part thereof) could be amplified from the genome in a polymerase chain reaction (PCR) using *hrpZ2* based primers.

In this report, the amplification and characterization of a *hrpZ2*-like fragment from the genome of *P. s.* pv. *syringae* NV will be discussed and arguments for designating this PCR product the putative harpin elicitor gene of *P. s.* pv. *syringae* NV will be presented. Many of the standard molecular biology procedures (e.g. transformation of competent *E. coli*, DNA isolation, restriction enzyme digestion and agarose gel electrophoresis) were discussed in detail in the previous chapter and will not be repeated here. Only the relevant details and significant variations on the standard procedure (where applicable) will be included in this presentation of related experiments.

MATERIALS AND METHODS

Cultivation of *E. coli* DH5α (pSYH10) and *P. s.* pv. *syringae* NV for DNA isolation

Plasmid pSYH10 (containing the *hrpZ2* gene of *P. s.* pv. *syringae* 61) was obtained from Alan Collmer (Cornell University, Ithaca, New York). The preparation and transformation of competent cells of *E. coli* strain DH5α with this plasmid, as well as the evaluation of the success of the transformation, have been discussed in detail in the previous chapter.

Clone pSYH10-a of the transformed bacterium was grown in LB medium, supplemented with 40 µg/ml ampicillin, for 16 hours at 37°C. Bacterial cells (2 x 50 ml) were pelleted by centrifugation (3 020 x *g* for 10 min) and plasmid DNA was isolated according to the manufacturer's instructions, using a Nucleobond AX PC-Kit 100 (Machery-Nagel). The final plasmid DNA precipitate was redissolved in warm (60°C) sterile double distilled water and stored at 4°C. The quantity and purity of isolated DNA (diluted 1/20) was assessed from absorbance readings at 260 nm (A_{260}) and 280 nm (A_{280}), using a Beckmann DU 650 spectrophotometer and mini (60 µl) cuvette. Since an A_{260} = 1 = 50 µg/ml double-stranded DNA (Maniatis *et al.* 1989), and a 1/20 dilution was used for spectrophotometric determinations, the undiluted DNA concentration (in µg/µl) could be obtained directly from the A_{260} value. To verify that the isolated plasmid DNA indeed consisted of plasmid pSYH10, a restriction digest with *Hind*III, followed by analysis of the reaction products on agarose gel electrophoresis, was performed as described previously.

P. s. pv. *syringae* NV (isolated from a nectarine tree) was obtained from the Infruitec (Institute for Fruit Technology, Stellenbosch) culture collection. The bacterium was grown in nutrient-yeast extract-glycerol broth (NYGB; 0.5 % (w/v) peptone, 0.5 % (w/v) yeast extract, 2 % (v/v) glycerol) at 27°C for 16 hours. Bacterial cells (6 x 1.5 ml) were pelleted by centrifugation and washed once in phosphate buffer (0.1 M, pH 6.5), after which total genomic DNA was extracted according to the method of Goodwin and Lee (1993). The protocol was modified slightly in that the microwave incubation was replaced by incubation in a water bath at 80°C for 20 min. The purity and concentration of the isolated genomic DNA was determined spectrophotometrically as described above.

Primer design

Oligonucleotide primers were designed to contain the terminal sequences of the *hrpZ2* gene. In order to be used effectively in a PCR, primers have to be designed in such a way that: (a) 20 to 30 bases are complementary to the target template sequence, (b) the forward and reverse primers have similar melting temperatures, (c) none of the primers are prone to the formation of stable secondary structures and (d) interactions between the two different primers, or between two primers of the same

kind (primer-dimers), especially involving 3' bases, are avoided as far as possible (Ausubel *et al.* 1995). For future cloning purposes, it was decided to include a common restriction endonuclease recognition sequence at the 5'-end of each primer. To satisfy the requirement of restriction endonucleases of having a number of bases flanking their recognition sequences for effective digestion, three randomly chosen bases were included at the terminal 5'-end of each primer.

All of the above requirements were best met by the following primers: 5'-ATA*GAATTC*ATGCAGAGTCTCAGTCTTAACAGCAGC-3' (forward primer) and 5'-TAA*TCTAGA*TCAGGCTGCAGCCTGATTGC-3' (reverse primer). The 36-mer forward primer contained (in the 5' → 3' direction) a random trinucleotide for restriction endonuclease binding (ATA), an *Eco*RI recognition site (*GAATTC*) and the first 29 bases of the coding strand of *hrpZ2* (starting with the start codon ATG). The melting temperature (T_m) of this primer was calculated with primer designer computer software to be 78°C. As a result of the much higher GC content of the 3'-end of the *hrpZ2* gene, a shorter reverse primer had to be selected to achieve a melting temperature similar to that of the forward primer. Seen in the 5' → 3' direction, the abovementioned reverse primer (29-mer, T_m = 77°C) consisted of a random trinucleotide for endonuclease binding (TAA), a *Xba*1 recognition sequence (*TCTAGA*) and 20 bases complementary to the last (3'-end) bases of the *hrpZ2* sequence (the codon closest to the *Xba*1 site being complementary to the stop codon of (TGA) of this gene).

The primers were obtained from the University of Cape Town Oligonucleotide Synthesis Service. For PCR purposes, 15 pmol/µl solutions were made of each primer. These diluted primer solutions proved to be stable at -20°C for up to three weeks.

PCR and agarose gel electrophoresis

In the choice of conditions for the PCR, a number of factors regarding the final concentrations of reagents and the thermal profile had to be considered (reviewed in Arnheim and Erlich 1992). As no information on the nucleotide sequence of and surrounding the amplification target (the harpin encoding gene of *P. s.* pv. *syringae*

NV) was available, a set of fairly "intermediate" reaction conditions were employed. The final concentration of $MgCl_2$ and annealing temperature chosen for the reaction were typical of more stringent reaction conditions, but would limit the amplification of non-specific primer binding products. Final concentrations of reagents (in 50 µl reaction volumes) were: 10 x PCR buffer (Advanced Biotechnologies), 1 x; thermostable DNA polymerase (Advanced Biotechnologies), 0.05 U/µl; dNTP's (Boehringer Mannheim), 0.2 mM each; $MgCl_2$ (Advanced Biotechnologies), 1.5 mM; primers, 0.5 µM each and 2-mercapto-ethanol (Bio-Rad), 17 mM. Total genomic DNA of *P. s.* pv. *syringae* NV (200 ng per 50 µl reaction) was used as template. As a positive control for amplification, pSYH10 plasmid DNA (200 ng/50 µl) was used as template. The template DNA was replaced with sterile double distilled water as a negative control.

The PCR's were carried out in a Minicycler™ (MJ Research) with an in-sample probe. The thermal profile consisted of an initial denaturation step (94°C for 3 min), followed by 36 cycles of 94°C (1 min), 65°C (2 min), 72°C (2 min) each, and a final elongation step of 5 min at 72°C. PCR products were identified by electrophoresis of 15 µl of each reaction product in a 1 % agarose gel in 1 x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) as described previously. Fragments generated by *Pst*1 digestion of λ-DNA (1 µg) were used as molecular mass markers. Ethidium bromide (0.33 µg/ml) was included in the gel for visualization of the electrophoresis result under ultra-violet (UV) illumination.

Partial sequencing of *P. s.* pv. *syringae* NV PCR product

Purification of PCR product

The *P. s.* pv. *syringae* NV PCR reaction product was purified for sequencing purposes from an agarose gel according to the following procedure. Of the final reaction 35 µl was run in 1 x TBE on a 0.8 % agarose gel containing ethidium bromide as described above. The ± 1 kilobasepair (kb) band was cut out under UV illumination and purified from the agarose using the method described by Tuatz and Renz (1983). The protocol was modified in that siliconized glass beads were used instead of silanized glass wool and the second freeze-step was carried out in the

presence of 0.1 volume 3 M sodium acetate and 3 volumes of ethanol, instead of 0.01 volumes of 1 M $MgCl_2$ and 10 % (v/v) acetic acid, and 2.5 volumes of ethanol.

Cycle sequencing

Partial sequencing of the purified product was carried out using the Δ*Taq* Cycle Sequencing Kit (US Biochemicals) and the same primers as for the PCR. This chain-termination (Sanger *et al.* 1977) sequencing strategy is performed in two steps, each of which is cycled to provide sufficient product for the sequencing of small amounts of template. The first (primer-labelling) step is carried out separately for the forward and reverse primers under conditions where the extension of each is limited to a few nucleotides, including some labelled ones. This is achieved through the use of only three of the four deoxynucleotide triphosphates (dNTPs), of which one is labelled radioactively. Each of the labelled primers are subsequently divided among the four chain termination reaction mixtures, containing increased dNTP concentrations and one dideoxynucleotide triphosphate (ddNTP) each (Tabor and Richardson 1987, Huibregtse and Engelke 1991, Ruano and Kidd 1991). The ladders of labelled target DNA fragments created separately from both ends of the template in this way, are subsequently separated by denaturing polyacrylamide electrophoresis.

The separate primer labelling reactions (final primer concentration, 10 nM) were both carried out according to the manufacturer's instructions in the presence of [α-^{35}S]dCTP (Amersham, 400 Ci/mmol) and no dATP. Based on the *hrpZ2* sequence, this would result in a forward primer extended for 7 bases, of which 3 would be radioactively labelled. Similarly, the resultant reverse primer would be extended with 3 bases, all of which would be labelled. A modified thermal profile, consisting of an initial denaturation (90 s at 94°C) and 50 cycles of 94°C (20 s), 48°C (30 s), 72°C (2 s) each, was followed for the labelling reactions. Labelling reactions were each split into the four subsequent ddGTP, ddATP, ddTCP and ddCTP termination reactions according to the manufacturer's instructions. The thermal profile for termination reactions consisted of an initial denaturation step at 95°C (30 s), followed by 50 cycles of 94°C (60 s), 65°C (60 s), 72°C (90 s). Reactions were stopped according to instructions.

Electrophoresis and visualization of sequencing reaction products

Electrophoresis of the reaction products was performed using a vertical EC160 DNA Sequencing System (E-C Apparatus Corporation) on a 35 cm x 43 cm x 0.25 mm polyacrylamide gel (6 % T, containing 8.2 M urea). A multiple V-tooth comb was used for the formation of sample loading wells. The gel was pre-electrophoresed in 1 x TBE to a temperature of 45°C. Termination reaction products were denatured for 2 min at 95°C, after which 5 µl of each of the four forward primer reactions and the four reverse primer reactions were loaded in adjacent lanes. Electrophoresis was carried out in 1 x TBE for 2 hours at 50 W (45°C). The sequencing gel was dried onto filter paper and exposed to X-ray film (Chromex) in a light-tight container at room temperature before being developed according to the standard procedure (5 min in developing solution, followed by a rinse in water and 3 min in fixer solution; both solutions from Protea Medical Suppliers). The partial sequence of the *P. s.* pv. *syringae* NV PCR product was read from the developed film.

Slot blot hybridization

Hybridization of the *P. s.* pv. *syringae* NV PCR product with the *Bst*X1 fragment of the *hrpZ2* gene was carried out using the non-radioactive digoxigenin (DIG) system (Boehringer Mannheim). The first step of this procedure involved preparation of the hybridization probe. This was achieved by restriction digestion of plasmid pSYH10, followed by purification and DIG-labelling of the *Bst*X1 fragment of *hrpZ2*. In the second step of this procedure, the probe was hybridized to the target DNA (immobilized on a membrane), washed stringently and the hybridization result visualized by chemiluminescence.

Preparation of probe

pSYH10 DNA was digested overnight with *Bst*X1 in SuRE cut buffer H (Boehringer Mannheim) at 45°C. The final reaction was separated on a 1 % agarose gel as described before. The gel slice containing the smaller of two fragments (3555 and 780 bp respectively) was cut out and the DNA purified from the agarose gel as described before. This *Bst*X1 fragment (100 ng) was denatured (10 min at 95°C) and labelled non-radioactively for 20 hours according to the random primed labelling

procedure, using the DIG DNA Labelling Kit (Boehringer Mannheim). In this procedure, random hexanucleotides, dNTP's, DNA polymerase I (Klenow enzyme, large fragment; from *E. coli*) and digoxigenin-11-deoxyuracil triphosphate (DIG-dUTP) were used to synthesize DIG-labelled DNA fragments complementary to each of the strands of the denatured *Bst*X1 fragment. The labelled double-stranded DNA (containing a DIG-dUTP (instead of a dTTP) every 20 to 25 nucleotides) was purified from the reaction mixture by ethanol precipitation according to the manufacturer's instructions. The concentration of this purified, labelled *hrpZ2-Bst*X1 probe was estimated at 1 ng/µl, using the DIG-kit for estimating the yield of DIG-labelled DNA (Boehringer Mannheim).

Slot blot hybridization

After denaturation for 10 min at 95°C, 25 µl of each final PCR reaction mixture (positive control, negative control and *P. s.* pv. *syringae* NV template reaction) were mixed with 0.1 volume of 20 x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0; supplemented with 0.1 % (w/v) SDS) and transferred with a slot blot apparatus onto a Hybond™-N⁺ membrane (80 cm², Amersham). DNA was fixed to the membrane by alkaline denaturation (3 min in 1.5 M NaCl, 0.5 M NaOH, followed by neutralization in 0.5 M Tris-Cl, 1.5 M NaCl, 1 mM EDTA for 3 min) and UV crosslinking (10 min). The membrane was pre-hybridized for 4.5 hours at 42°C in DIG EasyHyb solution (Boehringer Mannheim) and hybridized overnight at 42°C with the abovementioned DIG-labelled *hrpZ2-BstX*1 probe (3 ml). To remove excess and non-specifically bound probe, the membrane was washed twice in 2 x SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0; supplemented with 0.1 % (w/v) SDS) for 5 min at room temperature. This was followed by two stringent washes of 15 min each in 0.1 x SSC (0.15 mM NaCl, 1.5 mM sodium citrate, pH 7.0; supplemented with 0.1 % (w/v) SDS) at 68°C.

The hybridization result was detected according to the manufacturer's instructions, using the DIG Luminescent Detection Kit (Boehringer Mannheim). In brief, this involved blocking of the membrane to prevent the non-specific binding during the subsequent incubation with an alkaline phosphate conjugated anti-digoxigenin-antibody. Incubation with the substrate, Lumigen™ PPD, resulted in a chemiluminescent signal, which was detected on X-ray film (Chromex). The film was

exposed to the membrane for 2 hours at room temperature, after which it was developed as described previously for the visualization of sequencing reaction results.

RESULTS AND DISCUSSION

DNA isolation

Results of DNA isolation from bacterial cultures are given in Table 5-1. The A_{260}/A_{280} ratios of 1.826 and 1.919 respectively indicated that the DNA preparations contained very little contaminants (Maniatis *et al.* 1989). Restriction digestion of the *E. coli* DH5α (pSYH10) plasmid preparation with *Hind*III yielded two bands of approximately 3.4 and 0.9 kb in size (results not shown, see chapter 4, Fig. 4-7 for a similar result), confirming that the isolated plasmid was indeed pSYH10.

Table 5-1. Results of DNA isolations from bacterial cultures.

Bacterium	DNA isolated	A_{260}	A_{280}	A_{260}/A_{280}	[DNA] (μg/μl)
E. coli DH5α (pSYH10)	Plasmid DNA	1.329	0.7277	1.826	1.329
P. s. pv. *syringae* NV	Total genomic DNA	0.726	0.378	1.919	0.726

PCR Amplification of a ± 1 kb fragment from the *P. s.* pv. *syringae* NV genome

The PCR products obtained with the *hrpZ2* based primers, using pSYH10 plasmid DNA and *P. s.* pv. *syringae* NV total DNA respectively as templates, are shown in

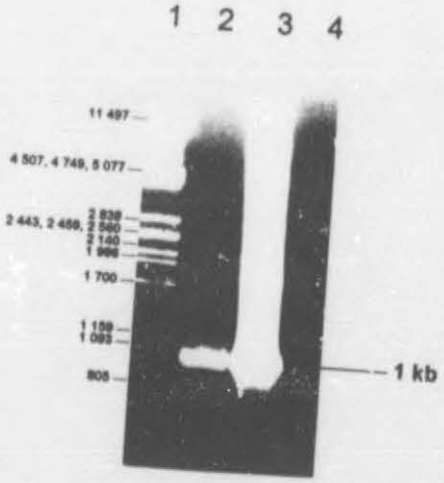

Fig. 5-1. Agarose gel electrophoresis of PCR products.

Lane 1: Molecular mass markers (*Pst*1 digest of λ-DNA), **lane 2:** Product of PCR with *P. s.* pv. *syringae* NV total DNA as template, **lane 3:** Positive control (plasmid pSYH10 used as template), **lane 4:** Negative control (no template DNA in PCR).

The PCR and electrophoresis was carried out as described in Materials and Methods. The sizes (bp) of marker fragments are given on the left.

Fig. 5-1. The most intense band (± 1 kb) in the pSYH10 reaction corresponded to an amplified *hrpZ2* gene (1026 bp). Less intense bands were the result of non-specific binding of primers to the plasmid. The only significant product of the *P. s.* pv. *syringae* NV reaction was the band corresponding in size to the *hrpZ2* gene.

This result indicates the presence of a DNA fragment in the *P. s.* pv. *syringae* NV genome that has the same size as the *P. s.* pv. *syringae* 61 *hrpZ2* gene. Since the amplification product was obtained under relatively stringent conditions regarding the $MgCl_2$ concentration and annealing temperature used in the PCR, it is evident that this *hrpZ2*-like fragment shares a reasonable degree of homology with the terminal 29 bases at the 5'-end and the terminal 20 bases at the 3'-end of this gene. The PCR was conducted in the presence of a high concentration of template DNA

(200 ng/50 µl reaction). However, the same result was obtained for a concentration series of template DNA (60-200 ng template DNA per reaction; results not shown). The high quantity of DNA was maintained in repetitions of the PCR, as the yield of specific product for subsequent experiments would be higher.

Partial sequencing of *P. s.* pv. *syringae* NV PCR product

Separation of the cycle sequencing reaction products (Fig. 5-2) revealed more information on homology between the amplified *P. s.* pv. *syringae* NV sequence and that of *hrpZ2*. No significant data could be obtained from the reactions using the for-

Fig. 5-2. Autoradiograph of cycle sequencing rection products separated by polyacrylamide gel electrophoresis.

Cycle sequencing and electrophoresis were performed as described in Materials and Methods. The four left-handed lanes on the autoradiograph correspond to the four reactions (G, A, T and C) carried out with the forward primer and the four right-handed lanes to the reverse primer reactions. Because the reverse primer is complementary to the coding strand of pSYH10, a band in the G lane would correspond to an A in the coding strand of the *P. s.* pv. *syringae* NV PCR product, provided that the orientation of the two sequences are the same. This was accepted to be the case in the interpretation of sequence data obtained from this experiment.

ward primer, which means that labelling of this primer (which had to be based on the *hrpZ2* sequence) did not occur as envisaged. However, strong signals were obtained in reverse primer reactions. This result indicates that homology between the amplified *P. s.* pv. *syringae* NV sequence and *hrpZ2* is less in the 5'-region than in the 3'-region.

As this was a preliminary sequencing reaction, the sequence of only 101 bases in the 3'-region of the amplified sequence could be read with confidence. When aligned in such a way as to obtain the largest degree of homology (see Fig. 5-3), the partial sequence obtained for the amplified *P. s.* pv. *syringae* NV region corresponded with bases 799 to 906 of *hrpZ2*, with the exception of 7 bases. These bases were absent in the *P. s.* pv. *syringae* NV sequence. In four of the seven cases, the "extra" base in the *P. s.* pv. *syringae* 61 sequence had a similar nucleotide next to it (i.e. GG, CC or AA). It is thus possible that the second base in these cases were overlooked in the *P. s.* pv. *syringae* NV sequence due to band compression on the sequencing gel (Barr *et al.* 1986, Mizusawa *et al.* 1986). The difference in (the remaining) three nucleotides between the compared sequences would imply that the resultant two proteins shared the same reading frame: a result which is expected in the light of the homology on the protein level reported previously for the harpins produced by these two strains (Appel *et al.* 1995, chapter 4 of this thesis).

Results from the sequencing experiment also coincide with a previous report that a functional C'-terminal of the *hrpZ2* product (harpin$_{Pss}$) is required for elicitor activity (He *et al.* 1993). Two repeated amino acid sequences (GGGLGTP and QTGT) in the C'-terminal of harpin$_{Pss}$ where shown to be essential for an elicitor active recombinant protein. The corresponding DNA sequences of one set of these repeats fell within the region that was aligned to the known fragment of the *P. s.* pv. *syringae* NV amplified region. A high degree of homology with a region, that is likely to be conserved because of its role in the activity of the protein product, indicated that the amplified *P. s.* pv. *syringae* NV region (or part thereof) constitutes the gene encoding the harpin of *P. s.* pv. *syringae* NV.

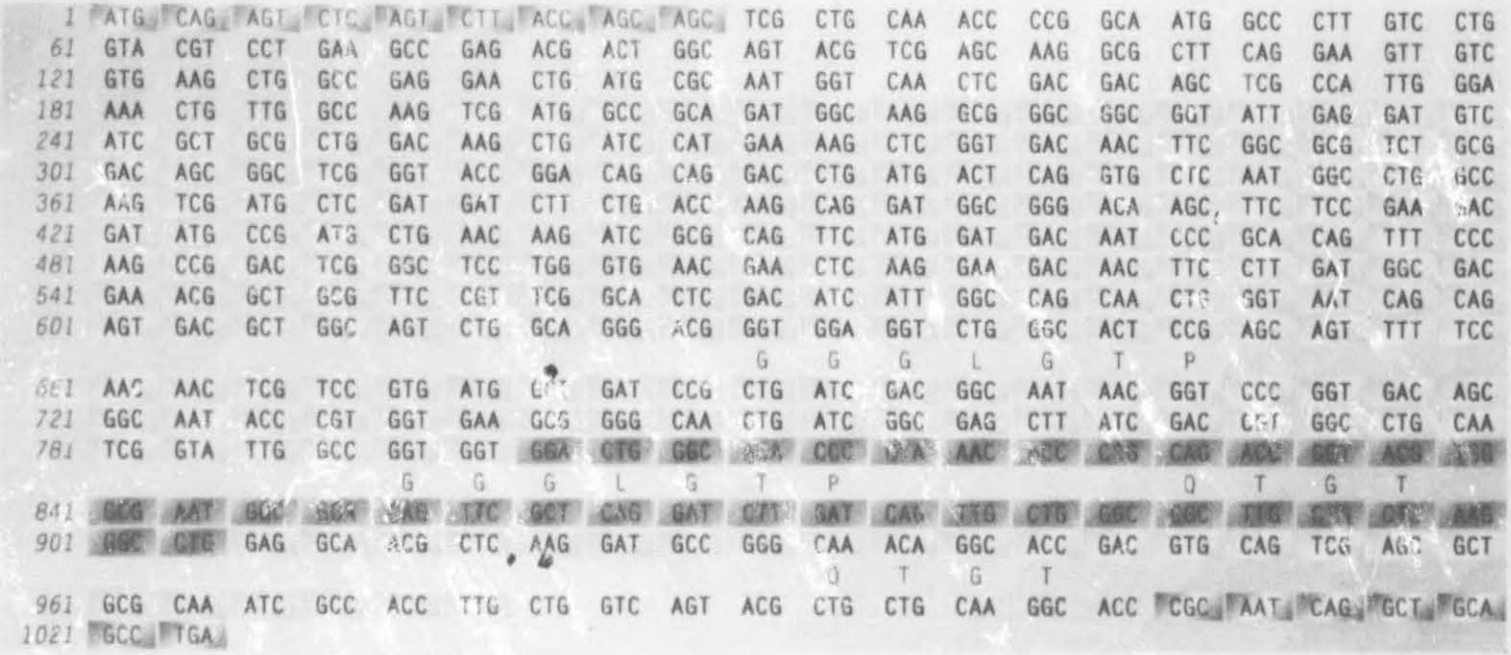

Fig. 5-3. Sequence homology between the *P. s.* pv. *syringae* 61 *hrpZ2* gene and the amplified *P. s.* pv. *syringae* NV region.

The above sequence of the *hrpZ2* gene and its characteristics were published previously (He *et al.* 1993). Amino acid repeats (GGGLGTP and QTGT) in the C'-terminal region that were found essential for elicitor activity are indicated under their corresponding DNA sequences. The partial sequence data obtained for the amplified *P. s.* pv. *syringae* NV fragment was aligned with bases 799 to 906 of the *hrpZ2* sequence (red). The two sequences were found to be identical, except for the bases indicated in blue in the *hrpZ2* sequence. When aligninig the two sequences in a way as to obtain maximum homology, these bases were absent in the amplified *P. s.* pv. *syringae* NV region. The segments of *hrpZ2* contained in the primers used for the PCR and cycle sequencing are indicated in green. The *Bst*X1 fragment used for slot blot hybridization has a yellow background.

Hybridization of the *P. s.* pv. *syringae* NV PCR product with the *Bst*X1 fragment of *hrpZ2*

The result of non-radioactive hybridization between the *P. s.* pv. *syringae* NV amplification product and the *Bst*X1 fragment (comprising 76% of the *hrpZ2* gene, from bases 199 to 978) is shown in Fig. 5-4. A strong hybridization signal was obtained under conditions of stringent washing. As the *Bst*X1 fragment does not contain the sequences of the primers used in the PCR, this result confirms that homology exists between the putative *P. s.* pv. *syringae* NV harpin gene and *hrpZ2* in areas other than where the primers annealed during the PCR (see Fig. 5-3).

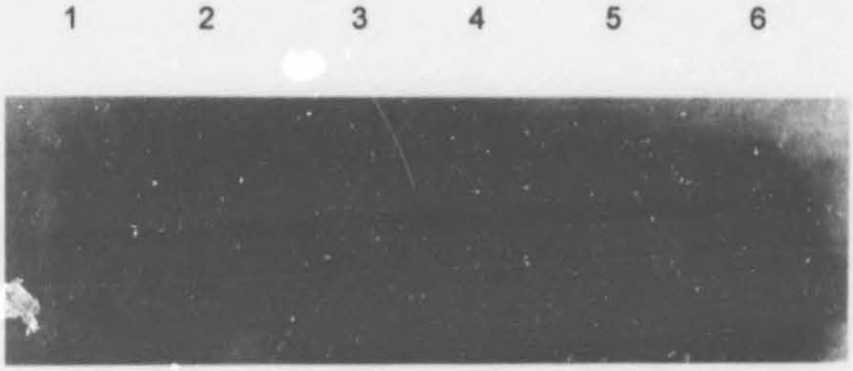

Fig. 5-4. Result of non-radioactive slot blot hybridization of PCR products with the DIG-labelled *Bst*X1 fragment of *hrpZ2*.

1 and 2: Negative control (no template DNA in the PCR), **3 and 4:** Positive control (pSYH10 plasmid DNA used as template for the PCR), **5 and 6:** Product of PCR with *P. s.* pv. *syringae* NV total genomic DNA as template.

In conclusion, the results of this study contribute to the existing evidence that the South African nectarine pathogen, *P. s.* pv. *syringae* NV, produces a harpin elicitor similar to the harpin of the wheat pathogen, *P. s.* pv. *syringae* 61 (Appel *et al.* 1995, chapter 4 of this thesis). The homology in size and partial sequence homology that

was demonstrated here between the 1.1kb *hrpZ2*-like DNA fragment amplified from the genome of *P. s.* pv. *syringae* NV in this study and the *hrpZ2* gene of *P. s.* pv. *syringae* 61 argues that the former (or part thereof) constitutes the gene encoding the harpin elicitor (or part thereof) produced by the nectarine pathogen. Cloning of this fragment into a suitable vector for over-expression and confirmation of the identity of its protein product is in progress and will be reported on at a later stage.

CHAPTER 6

DISCUSSION

Two objectives were set for this study, namely to (a) isolate and purify the harpin elicitor produced by *Pseudomonas syringae* pv. *syringae* NV and (b) to locate the gene encoding the nectarine pathogen harpin. Seen in the light of the literature published in this field over the past decade, the approach chosen to meet the first of these objectives was perhaps somewhat ambitious. The failure of all previous attempts to purify the elusive bacterial elicitor of the hypersensitive response using standard biochemical methodology (Hutcheson *et al.* 1989, Kado 1994) (mis)led some researchers to reject the notion of a secreted bacterial elicitor, whose recognition by a plant receptor mediates the nature of a plant-pathogen interaction (Kado 1994). The purification of recombinant harpins (Wei *et al.* 1992a, He *et al.* 1993, Panopoulos 1995) not only proved the existence of such molecules; the characteristics of harpins revealed in these studies also explained why they were so difficult to isolate biochemically. More importantly, these studies demonstrated why the strategy presented in this study has merit. The approach followed towards the purification of the *P. s.* pv. *syringae* NV harpin was therefore not an unattainable one; yet experimental difficulties and the lability of the protein rendered it impractical for large-scale or routine purification purposes.

The approach followed to amplify the *P. s.* pv. *syringae* NV harpin encoding gene from its genome proved to be less ambitious. DNA homology between the *hrp* loci of different *P. syringae* pathovars has been widely demonstrated (Panopoulos *et al.* 1985, Lindgren *et al.* 1986, 1988, Huang *et al.* 1991, Hutcheson *et al.* 1994). The results obtained in the first part of this study specifically confirmed homology between the harpin encoding gene of *P. s.* pv. *syringae* NV and the well characterized *hrpZ2* gene of *P. s.* pv. *syringae* 61. Although convincing evidence for the identity of the 1 kb *hrpZ2*-like DNA fragment amplified from the *P. s.* pv. *syringae* NV genome as the harpin encoding gene of this strain was presented, it can only be confirmed without doubt in the following series of experiments: (a) cloning of the PCR product in a suitable expression vector, (b) purification of the recombinant protein product, (c) assessment of the HR phenotype of this recombinant protein

using the HR test in tobacco, (d) production of antibodies against this protein and (e) Western blot analysis of elicitor active *P. s.* pv. *syringae* NV fractions with the antiserum to confirm that the same protein is secreted by the native bacterium. A prerequisite for this series of experiments is, however, the complete sequencing of the amplification product, in order to ensure that it be cloned in the correct reading frame in a suitable expression system. It is envisaged that this will be undertaken in future.

The investigation into the production of a harpin elicitor by a strain of *Pseudomonas syringae* does not represent a new field of study. The long-term objective of the project (of which this study formed the basis), namely to apply a purified harpin in the screening of South African commercial stone fruit cultivars for resistance against bacterial canker, is however, a novel one. It was born from the almost exceptional destructiveness of the disease in South Africa and renders the emphasis of our (South African) research in the field of *hrp* genes and their role in plant-host pathogen interactions unique and more applied, compared to efforts in this field by American and European research groups. The value of the work presented in this thesis must thus also be evaluated in terms of the long-term project. Although neither of the specific objectives set at the beginning of this study were completely fulfilled, a sound basis for future research efforts has been established. Future research, to be undertaken jointly by the Department of Biochemistry, University of Stellenbosch and the Agricultural Research Council's Institute for Fruit Technology (Infruitec), will address the following aspects:

1. Complete sequencing of the *hrpZ2*-like *P. s.* pv. *syringae* NV DNA fragment, with the view towards its cloning and expression in a specialized protein purification system (such as the pMAL™ system, New England Biolabs).

2. Assessment (either on transcriptional or translational level) of the induction of systemic plant defence mechanisms (e.g. phytoalexin production, the induction of peroxidases, chitinases and β-1,3- glucanases) by commercially important South African stone fruit cultivars upon challenge with the purified harpin of *P. s.* pv. *syringae* NV.

3. The production of highly specific antibodies against the nectarine pathogen harpin, for use in *in situ* hybridization studies aimed at elucidating the time course of harpin expression and the targeting of this molecule in plant tissue.

These proposed studies are aimed primarily at the identification of biochemical "markers" for resistance against bacterial canker, to be applied as possible selection criteria in future stonefruit breeding programmes.

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